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INFLUENCE OF SIMULATED ALTITUDE ON RESISTANCE-SUSCEPTIBILITY TO *S. TYPHIMURIUM* INFECTION IN MICE*

L. Joe Berry and Roland B. Mitchell†

Since the days of Pasteur recognition has been given to the changes in resistance to infection that may accompany conditions of stress. Just as Pasteur's chickens contracted anthrax after they had been immersed in cold water, so may other animals show changes in susceptibility to disease after fatigue (Nungester) and dietary restrictions (Clark *et al.*). In some cases, however, the change in resistance may not always be in the direction one might anticipate and the ultimate result may depend upon the route of infection as well as upon the specific pathogen selected to demonstrate the effect. Even though these relationships are almost as old as the science of bacteriology, little work has been devoted to elucidating the mechanism by means of which the response to infection is altered. This is not an easy experimental task since the differences with which the investigator works may demand careful and repeated verification. Nevertheless, the knowledge gained by such studies in this age when the human being is subjected to increasing stresses seems to warrant the effort. With this in mind, the experiments to be described in this report were undertaken.

It is an accepted fact that continued exposure to altitude induces polycythemia in animals and man. In view of Berry and Haller's report that mice made anemic by blood loss are more resistant to artificially induced infections, the question of the immune status of animals with polycythemia arises. Since Berry has shown that the enhanced defense of anemic animals can be related, at least in part, to the increased phagocytic activity of

* From the Department of Biology, Bryn Mawr College, Bryn Mawr, Pennsylvania, and Department of Microbiology, USAF School of Aviation Medicine, Randolph Field, Texas. This study was supported at Bryn Mawr College by funds provided under contract AF 33(038)–17842 with the USAF School of Aviation Medicine, Randolph Field, Texas. Received for publication May 20, 1953.

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blood neutrophiles and of macrophages in peritoneal exudates, it seemed of interest to evaluate the activity of the cellular defense of animals adapted to altitude. A *priori*, the same type of response to hypoxia of altitude and to hemorrhage might be anticipated. Both conditions are accompanied initially by an impairment of oxygen transport, for which there may or may not be adequate compensation, and subsequently by increased erythropoietic activity. The present report is, therefore, concerned with the results of a study designed to determine the susceptibility of mice exposed to simulated high altitude to infection with *S. typhimurium* and to compare their response to that of both normal and anemic mice. It is shown that the stress of altitude and the stress of blood loss lead to different types of response to infection but in neither case is it possible to establish any marked change in the body defense mechanisms usually associated with resistance to bacterial diseases.

Methods. Decompression chambers patterned after a design from the School of Aviation Medicine were fabricated in the laboratory shops at Bryn Mawr College. The round metal base containing the connections for the Cenco Hyvac pump, the mercury manometer, and the two air inlet valves, one a commercially available needle valve, was covered with a Pyrex aquarium jar with the rim ground plane. The electrical control circuit was designed by Professor Walter C. Michels, Department of Physics, Bryn Mawr College, and is shown in figure I. This system can be operated continuously for weeks on end with the pressure inside the chamber controlled to within a few millimeters of mercury. Each chamber was large enough

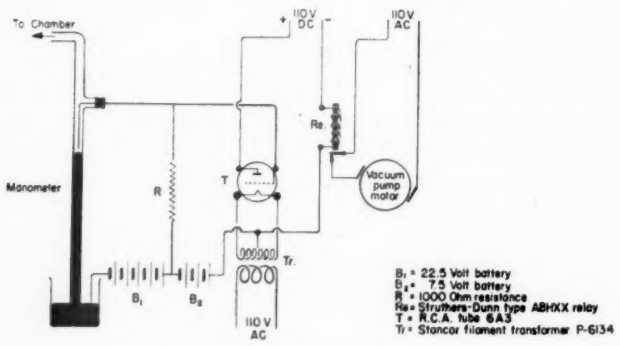

FIG. I. Circuit diagram for regulating pressure inside decompression chambers.

to house from 15 to 18 mice. At the beginning of an adaptation period, the pressure was set equivalent to an altitude of about 15,000 feet (the elevation of Bryn Mawr, Pennsylvania, is about 400 feet) and it was lowered each day by an amount equal to an elevation of 1,000 feet until the pressure at 20,000 feet was reached. This pressure was then maintained for the remainder of the adaptation period, usually a period totaling three weeks. The chambers were brought to atmospheric pressure each day in order to replenish the food and water supply and to provide clean bedding for the cages. This required only a few minutes.

Mice were made anemic by standardized hemorrhage according to the method first described by Tabor *et al.* At each bleeding 0.4 ml. of blood was removed from each mouse. This is equivalent to approximately 2 per cent of body weight in most of the mice employed. All animals were bled thrice weekly for a total of nine bleedings, unless otherwise specified.

Mouse typhoid was induced in the animals by inoculating them with a saline suspension of *S. typhimurium*. In most of the experiments, it was injected intraperitoneally but for one experiment intrastomachal injection was used. For the latter procedure, the bevel was removed from a 25 gage, one inch, hypodermic syringe needle and a drop of solder in the form of a smooth round ball was attached to the end. The distal one-quarter inch of the needle was bent to a 45 degree angle to make it easier to pass directly down the esophagus. The cells were cultured in brain heart infusion broth (Difco) for 18 hours at 37 degrees C. At the end of this time, 0.1 ml. of the culture was transferred to 9.9 ml. of sterile infusion broth and this was incubated at the same temperature for 5 hours. Repeated growth studies showed essentially a constant number of 750,000,000 viable bacteria per ml. in the 5 hour culture. Using this number as the standard, dilutions were made with 0.9 per cent sodium chloride solution (normal saline) to give the concentration of bacteria which preliminary experiments had established as appropriate for the particular experiment. The strain which was used throughout this work was originally obtained as an isolate from a mouse dead of typhoid in the laboratories of the Department of Microbiology, USAF School of Aviation Medicine.

In order to evaluate the phagocytic ability of cells in the spleen of mice from the different experimental groups, 0.2 ml. of thorotrast (a colloidal suspension of thorium dioxide prepared by the Heyden Chemical Corporation) was injected into the tail vein and 24 hours later the animals were sacrificed. Chemical analysis for thorium dioxide was carried out on the dried organs following the procedure of Gordon and Katsh, a modification of the method of Gaunt and Wright and of Harrington and Huggins. Radio analysis was also used to determine the amount of thorium in the same organs of different mice. Thorium undergoing radioactive decay gives off an alpha particle which can be detected by a Geiger tube with a thin-walled quartz window. The low penetrability of matter by alpha particles demands that the sample to be analyzed be uniformly distributed in a thin layer in front of the window of the Geiger tube. This was accomplished by drying the organs thoroughly in a hot air oven at about 100 degrees C., grinding

them in a small agate mortar with an agate pestle, transferring the powder with the help of a camels hair brush to a stainless steel pleget which was then placed a fixed distance from the window of the Geiger tube. A Berkeley Scaler was used to record the impulses from the tube in a laboratory where the background count was about one per 1.5 seconds. In the experiments in which the reticulo-endothelial system of the mice was blocked, the same volume of thorotrast was injected intravenously the day preceding the administration of the artificial infection.

Blood counts were made, for the most part, on the blood of animals sacrificed for thorium analyses. Such animals were killed by decapitation and the blood was collected in small paraffined watch glasses. It was then used quickly for the erythrocyte and leucocyte counts and hemoglobin was determined colorimetrically by adding 20 cubic millimeters of blood to 10 ml. of distilled water contained in a test tube. Some blood counts were made with tail blood by using the small diluting pipettes specifically designed for the purpose.

Mice were immunized against *S. typhimurium* by injecting 0.5 ml. of heat-killed phenolized suspension of bacteria containing 750,000,000 cells per ml. The organisms were cultured on brain heart infusion slants, harvested in normal saline containing 0.5 per cent phenol and killed by heating for an hour at 60 degrees C. After thorough shaking, counts were made in a Petroff-Hauser chamber and the final dilution was made to give the desired number of organisms per ml. All mice were injected intraperitoneally one week prior to the day on which they were infected with the same organism against which they had been immunized.

The mice were housed in galvanized iron cages large enough for about ten animals. Wood shavings were placed in the cages for bedding. Purina dog chow checkers and water were available at all times. The CF-1 strain of mice, obtained from Carworth Farms, were used except for one series of experiments in which Webster's inbred strains from the Rockefeller Institute were employed. Both males and females were tested separately in early experiments. Since no difference was detected in their response to infection, females were used thereafter. This choice was made in order to avoid losses due to injuries suffered in the inevitable fights between older males. Mice at the time of infection weighed between 20 and 25 gms., with but few exceptions.

Results

1. *Changes in the blood picture of mice subjected to simulated high altitude and to standardized hemorrhage.* Table 1 summarizes the results of total erythrocyte counts, hemoglobin content and total leucocyte counts in the blood of mice from groups used in this investigation. Average values, with standard deviations, for 44 control mice are given in line one. Also shown is the range over which the individual values spread. When the averages for

normal mice are compared with those given by Fekete it is seen that the erythrocyte numbers are in close agreement while the leucocyte counts are slightly higher in our mice and the hemoglobin is lower. The latter difference presents something of an unresolved problem. The value of 10.9 ±1.0 gm. per cent in table 1 is based on colorimetric determinations in which 15 gm. is equivalent to 100 per cent hemoglobin. Fekete's value is 97 per cent on the Sahli scale where 17.3 gm. are equivalent to 100 per cent. Since the blood of the normal human being contains about 15 gms. of hemoglobin, as determined by our method, it must be concluded that our mice have a hypochromic anemia, a conclusion that differs from Fekete's. Nevertheless, the value listed in table 1 serves as a useful frame of reference for changes in the

TABLE 1

Average Number of Leukocytes and Erythrocytes and Average Hemoglobin Content of Blood from Control Mice, from Mice after Different Periods of Exposure to and Recovery from Altitude, and from Mice Subjected to Standardized Hemorrhage.

Group and Description	No. of Mice	Leukocytes per cubic mm. Average	Leukocytes per cubic mm. Range	Erythrocytes per cubic mm. (in millions) Average	Erythrocytes per cubic mm. (in millions) Range	Hemoglobin in gms. per 100 ml. blood Average	Hemoglobin in gms. per 100 ml. blood Range
1. Control	44	6,200± 2,859	2,200– 17,300	10.18 ±1.11	7.20– 12.53	10.9 ±1.0	8.6– 13.5
2. Altitude 7 days	5	9,870±	4,000–	11.43	10.04–	13.5	12.1–
1 day recovery		6,193	19,700	±1.45	13.73	±1.2	14.3
3. Altitude 14 days	15	7,970±	2,700–	13.15	10.68–	14.1	12.4–
1 day recovery		4,306	14,400	±1.15	15.10	±1.2	16.2
4. Altitude 14 days	8	5,250±	3,300–	10.81	9.05–	11.0	7.8–
14 days recovery		2,429	10,400	±0.79	12.00	±1.7	13.9
5. Altitude 21 days	36	5,885±	2,700–	14.51	10.35–	15.0	12.0–
1 day recovery		2,623	11,100	±1.63	17.90	±1.3	16.9
6. Altitude 21 days	10	7,170±	2,600–	14.34	12.25–	15.1	13.3–
3 days recovery		2,800	11,450	±1.61	17.75	±1.0	16.4
7. Altitude 21 days	8	9,375±	5,750–	12.02	10.83–	14.5	13.5–
7 days recovery		2,479	12,400	±1.04	13.08	±0.5	15.0
8. Altitude 21 days	6	8,108±	5,050–	12.85	10.30–	11.6	9.5–
10 days recovery		2,046	11,350	±1.66	13.80	±1.5	13.1
9. Altitude 21 days	5	10,150±	8,050–	9.64	8.60–	10.8	10.4–
14 days recovery		2,275	13,100	±0.96	11.13	±0.5	11.6
10. Altitude 21 days	8	4,878±	2,900–	9.85	8.69–	10.4	9.1–
21 days recovery		1,672	7,100	±1.00	11.73	±0.9	11.6
11. Anemic—1 day	7	7,800±	4,500–	5.24	4.78–	4.7	4.1–
after 6th bleeding		4,940	16,000	±0.28	6.00	±0.4	5.9
12. Anemic—day	10	....	....	4.47	3.62–	...	...
of 7th bleeding		....	....	±0.80	6.10	...	...
13. Anemic—1 day	44	8,200±	1,800–	5.68	4.10–	5.8	3.7–
after 9th bleeding		4,222	18,200	±0.75	6.86	±0.9	8.1
14. Anemic—3 days	8	5,269±	3,200–	8.15	5.30–	7.4	6.8–
after 9th bleeding		1,606	8,750	±1.51	10.13	±0.7	8.6

blood accompanying the stress of simulated high altitude and of standardized hemorrhage.

Mice subjected to a simulated high altitude undergo blood changes, as seen in table 1, which show that (a) erythrocyte numbers and hemoglobin values after 21 days at altitude, line 5, exceed those observed after 14 days, line 3, which, in turn, exceed those found after 7 days, line 2; (b) recovery of a normal blood picture in mice that have been at altitude for either 14 days or 21 days is complete at normal atmospheric pressures after 14 days, lines 4 and 9, or after 21 days, line 10, but not after 10 days, line 8; (c) the increase in erythrocyte number and in hemoglobin is about 50 per cent above normal values following two or three weeks at altitude and (d) no significant change in leucocyte number accompanied the hypoxic stress of simulated high altitude.

An examination of that part of table 1 pertaining to the results of blod counts with anemic mice shows (a) that the severity of the anemia one day after the sixth hemorrhage is as severe as it is one day after the ninth hemorrhage; (b) the lowest average count appears in mice on the same day that they receive the seventh hemorrhage; and (c) three days after the ninth hemorrhage the erythrocyte total and hemoglobin value show that recovery is rapid and should be essentially complete within a week. This is in agreement with previous observations for the rat (2). A comparison of the blood picture of anemic mice with control mice shows that the erythrocyte number and the hemoglobin content are reduced about 50 per cent by the hemorrhages while the leucocyte number is unaltered.

2. *Response of altitude mice to intraperitoneal infection with S. typhimurium compared to that of normal mice and anemic mice.* Control mice, mice subjected to the stress of high altitude for three weeks, and mice made anemic by nine standardized hemorrhages were infected intraperitoneally with 250,000 cells of *S. typhimurium* contained in 0.5 ml. of suspension. The injections were given after the altitude mice had been at normal atmospheric pressures for one day and, for anemic mice, one day after the ninth bleeding. The combined results of duplicate experiments are presented graphically in figure II. Each experiment gave comparable mortality curves but, in order to have larger numbers of animals with which to calculate by the Chi

square test the significant differences, the results of the two experiments were combined. An examination of the curves in figure II makes it apparent that (a) the altitude mice are the most susceptible of the groups from the fourth day post-infection to the end of the experiment but the greater susceptibility is statistically significant from the fourth through the eighth day. (b) The altitude mice have a higher death rate than the control mice on the fourth and fifth days while the control mice have the higher death rate on the sixth day. Beyond the sixth day, the altitude mice die at almost the same rate as the control and the anemic mice. (c) The anemic mice are more resistant than the control mice from the sixth day on but the greatest difference, statistically, is from the sixth through the ninth day. This greater resistance to infection with *S. typhimurium* confirms earlier results. (d) Anemic mice are statistically more resistant than altitude mice from the fourth day through the twelfth day post-infection.

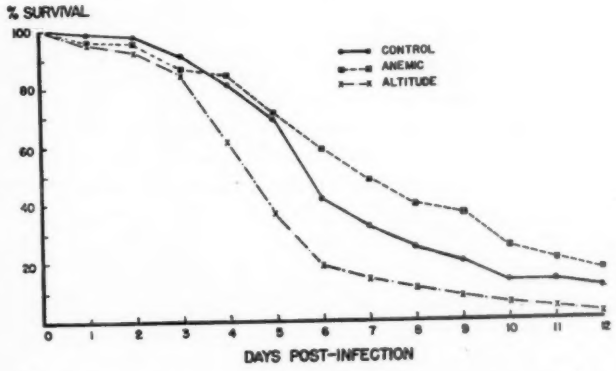

FIG. II. Survival curves of groups of mice infected intraperitoneally with 0.5 ml. of a suspension of *S. typhimurium* containing approximately 250,000 cells. Each curve is the average of duplicate experiments involving a total of 95 altitude mice, 95 anemic mice and 84 control mice.

3. *Comparison of the phagocytic activity of the reticulo-endothelial system in the different groups of mice as revealed by uptake of thorotrast in spleens.* In order to determine whether or not

some demonstrable change in the cellular defense accompanied the stress of altitude and of hemorrhage, and hence alters the susceptibility to infection, intravenous injections of thorotrast were followed, as previously described, by chemical and radioactive analyses of spleens. The results are presented in table 2 as average values with standard deviations. The following facts are established: (a) The spleens from anemic mice are slightly more than twice the size of normal spleens as shown by wet weight, dry weight, and ash weight. Histological preparations of a few of these spleens failed to reveal any cellular structures not usually seen in normal spleens but a searching analysis was not made. (b) The ratio of dry weight to wet weight for spleens from anemic mice (57.8/219.9=0.26) compared to control mice (25.5/106.1=0.24) and the ratio of ash weight to dry weight for spleens from anemic mice (13.4/57.8=0.23) compared to control mice (5.0/25.5=0.20) agree so closely, the increase in size of the spleens in anemic mice must not be due to fluid accumulation alone but rather to an accumulation of substances capable of contributing to the dry weight and ash weight to the same extent as the cells normally present. The histological appearance of the spleens from anemic mice is in agreement with the inference drawn from weight data alone. (c) No change in size of the spleen accompanies adaptation to a simulated high altitude for a period of three weeks as shown by wet weight, dry weight, and ash weight. (d) The uptake of thorotrast by the spleens of anemic mice is approximately twice that of the spleens of control mice and of altitude mice. The uptake of thorium per unit of spleen weight is the same in mice of all groups regardless of the type of weight employed for the calculation. (e) The determination of thorium by chemical method gives values from 77 per cent to 82 per cent of those obtained by Geiger counts. This difference approximates but exceeds the estimated combined error of the two methods. However, the close agreement in the percentage difference of thorium uptake as measured by the two techniques for each of the three groups of mice, *i.e.*, the 77 per cent to 82 per cent) argues against a random error of method and points to a real difference in the values obtained by the two procedures. Since some difficulties were experienced with the chemical method, particularly in redissolving the washed tho-

rium sulfate precipitate, greater confidence is placed in the analyses by Geiger counts.

4. *Response of altitude mice, anemic mice, and control mice to infection with S. typhimurium following blockage of the reticulo-endothelial system by intravenous injection of thorotrast.* The importance of the cellular defense in overcoming an artificially induced infection can be evaluated (Rischke) by first injecting an agent capable of overloading phagocytic cells and thereby rendering them less active. A variety of substances has been used for this purpose but, in recent years, thorotrast (Hanke) has come into prominence. Since the spleen of mice exposed to altitude removes thorotrast from the circulating blood in amounts equal to that of control mice (see table 2), the greater susceptibility of

TABLE 2

Uptake of Thorium Dioxide by Spleens of Altitude, Anemic, and Control Mice 24 Hours after Intravenous Injection of Thorotrast as Determined by Radioactive and by Chemical Analyses.

Experimental Group	Weights of Spleens						Thorium Dioxide Uptake in Mg. as Determined by			
	No.	Wet Wt.	No.	Dry Wt.	No.	Ash Wt.	No.	Radioactive Analysis	No.	Chemical Analysis
Altitude ...	24	102.0	42	25.0	13	6.2	20	5.6	21	4.5
		±24.6		±7.0		±2.8		±2.4		±1.4
Control	34	106.1	49	25.5	15	5.0	16	5.1	17	4.2
		±24.3		±7.7		±3.4		±2.4		±2.2
Anemic	23	219.9	47	57.8	16	13.4	16	11.2	27	8.6
		±52.9		±18.3		±11.4		±4.3		±4.3

these animals to infection is not to be attributed to an impaired cellular defense insofar as the results of the previous section permit a conclusion. Similarly, the increased activity of the enlarged spleen of anemic mice would account, at least in part, for the greater resistance of these animals to infection. In order to gain additional knowledge about the part played by the cellular defense in the response of altitude and anemic mice to infection, the experiments now to be described were undertaken.

All mice used in this experiment were injected intravenously with 0.2 ml. thorotrast approximately 24 hours prior to infection. The number of bacteria with which these animals were then infected intraperitoneally had been determined in a series of preliminary tests with mice whose reticulo-endothelial system had

been similarly blocked. Five thousand bacteria contained in 0.5 ml. of suspension gave a mortality curve approximating the one shown for control mice in figure II, infected with 250,000 cells. The ability of thorotrast to lower the resistance of mice is evidenced by the relative number of bacteria required for similar mortality.

The survival curves of each group of mice infected intraperitoneally with 5,000 cells of *S. typhimurium* the day after the injection of 0.2 ml. of thorotrast are given in figure III. The follow-

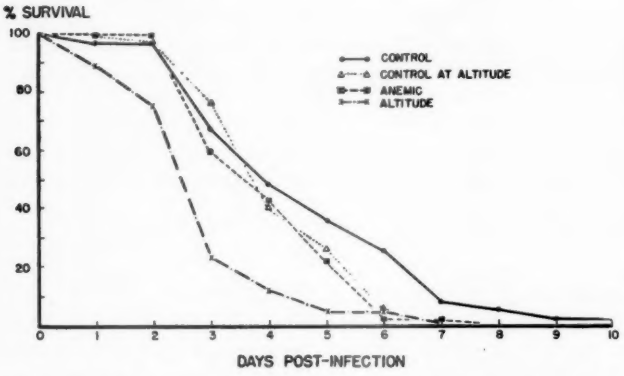

FIG. III. Survival curves of 47 control mice, of 48 control mice subjected to altitude immediately after infection, of 38 altitude mice, and of 58 anemic mice. All were injected intravenously with 0.2 ml. of thorotrast 24 hours prior to intraperitoneal infection with 5,000 *S. typhimurium* cells suspended in 0.5 ml. of saline.

ing relationships are evident: (a) Altitude mice are significantly more susceptible than control mice from the second day post-infection through the sixth day, beyond which time there were not enough survivors in either group to make the difference significant. (b) The survival of anemic mice was essentially identical to that of control mice for the first four days post-infection but by the sixth day they were significantly more susceptible. This is opposite to the results presented graphically in figure II. (c) Anemic mice are more resistant than altitude mice even when the activity of the cellular defense is impaired. This suggests that changes yet to be explained are responsible. (d) Mice subjected to simulated high altitude for the first time immediately after in-

fection and maintained there throughout the post-infection period have a survival curve paralleling that of anemic mice. A greater significant susceptibility is not manifest until the sixth day. This may indicate the time necessary for the appearance of the condition responsible for the greater susceptibility of mice at altitude.

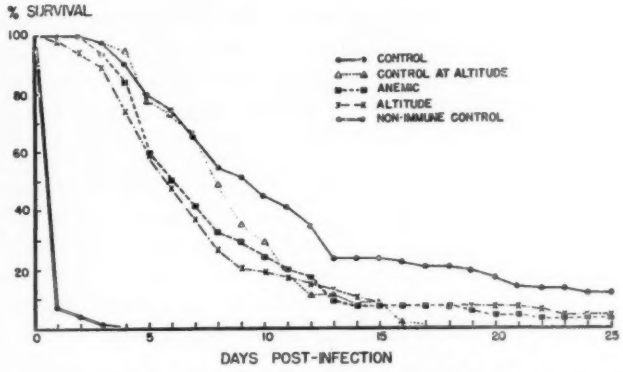

FIG. IV. Survival curves of 75 immunized control mice, of 72 immunized control mice subjected to altitude immediately after infection, of 65 immunized altitude mice, of 65 immunized anemic mice, and of 65 nonimmune control mice. All were infected intraperitoneally with 0.5 ml. of a saline suspension of *S. typhimurium*, containing about 2,500,000 cells.

5. *The response of altitude mice, anemic mice, and control mice to infection with S. typhimurium following immunization against the same organism.* A single immunizing injection was given to all mice used in this experiment one week prior to the day on which they were infected. Mice adapting to altitude were in the decompression chambers for six of the seven days and mice being hemorrhaged were bled three times after the immunizing injection was given. One of us (RBM), in unpublished observations, found the same agglutination titers formed in mice during exposure to altitude as at normal pressures. Similar results were obtained by Sax in mice undergoing standardized hemorrhage compared to normal mice. On the basis of these observations, it would be assumed that the comparative response to infection in such mice would be unaltered by immunization. Before the actual tests could be conducted, however, it was necessary to deter-

mine the number of bacteria required for mortality in normal immune mice similar to that in figure II for control mice. The number was 2,500,000 cells contained in 0.5 ml. of suspension.

The survival curves for the infected immunized mice are given in figure IV. The following facts are established by these results: (a) Altitude mice are significantly more susceptible than control mice from the fourth through the nineteenth days post-infection. Beyond this time, the differences are not significant. (b) Anemic mice are remarkably similar to altitude mice in their response, the two curves running close together throughout most of the post-infection period, thereby making these animals significantly more susceptible than control mice. (c) The control mice placed at altitude after infection are like control mice for the first week but by the ninth day they are significantly more susceptible than control mice and remain so until the last animal died on the seventeenth day. (d) The non-immune control mice were all dead on the fourth day but at the end of the first day 60 out of 65 mice were dead. This is a dramatic demonstration of the prophylactic value of immunization.

6. *Change in susceptibility to S. typhimurium accompanying exposure to simulated altitude in Webster's inbred bacterial resistant and bacterial susceptible strains of mice.*[1] By selective breeding, Webster obtained two strains of mice, one resistant and the other susceptible to *S. enteriditis* infection. He subsequently divided each of these strains into those that were virus resistant and those virus susceptible. He thus emerged with four strains of mice which possessed all possible combinations of bacterial and of virus resistance and suceptibility: (a) bacterial resistant and virus resistant (BRVR), (b) bacterial resistant and virus susceptible (BRVS), (c) bacterial susceptible and virus resistant (BSVR), and (d) bacterial susceptible and virus susceptible (BSVS). When these animals are infected with the proper number of bacteria the BR mice show more than 90 per cent survival and the BS mice show more than 90 per cent mortality from salmonellosis. As table 3 shows, there are relatively small differences in the phagocytic activity of blood neutrophiles from mice

[1] These mice were very generously supplied by Dr. Howard A. Schneider, Rockefeller Institute for Medical Research, to whom we express our sincere appreciation.

of the different groups as determined by the *in vitro* technique of Boerner and Mudd or in the ability of their spleens, livers, or lungs to ingest thorotrast, as determined by chemical analysis. Similarly, there is no difference in the ability of these mice to produce agglutinins against *S. typhimurium* (Mitchell). As far as these studies permit, one must conclude that neither the cellular defense nor the humoral defense is significantly different in the BR and BS mice.

TABLE III

Evaluation of the Cellular Defense of Webster's Genetically Resistant and Susceptible Mice

Experiments Conducted	STRAIN OF MICE BRVR No.	BRVR Average	BRVS No.	BRVS Average	BSVR No.	BSVR Average	BSVS No.	BSVS Average
Dry Wt. of Spleens.....	7	24.0 ±1.1	7	21.8 ±0.7	7	19.5 ±0.7	6	21.5 ±1.6
ThO_2 in Mg. per Spleen.....	7	8.0 ±1.0	7	7.3 ±0.4	7	7.9 ±0.5	6	7.5 ±0.5
Dry Wt. of Livers	5	499 ±28	6	386 ±22	2	390 ±8	4	323 ±8
ThO_2 in Mg. per Liver.....	5	16.9 ±7.3	6	26.3 ±4.8	2	22.8 ±6.0	4	20.7 ±3.9
Dry Wt. of Lungs.....	5	40.6 ±8.9	6	56.3 ±6.0	2	40.9 ±1.8	5	57.2 ±4.3
ThO_2 in Mg. per Lung.....	5	4.6 ±1.6	6	4.1 ±1.3	2	3.0 ±0.0	5	7.0 ±5.1
Percent of Blood Neutrophiles Phagocytically Active	16	34.4 ±5.1	14	38.0 ±4.6	14	44.3 ±4.8	16	38.6 ±4.6

Since these mice have now been inbred at the Rockefeller Institute for 25 to 35 generations, they constitute greater genetic homogeneity than mice normally employed for experimental purposes. In their studies of the effect of nutrition on natural resistance to infection, Schneider and Webster found that neither BR nor BS mice show any difference in their response to infection on diets which alter the resistance to salmonellosis of outbred mice like the CF–1 mice used in this work. By exposing groups of BR and BS mice to simulated altitude for three weeks before comparing their response to *S. typhimurium* infection with corresponding control mice, it was possible to establish whether the greater susceptibility of the altitude mice seen in figures II, III, and IV is dependent upon a genetic heterogeneity as it is in some dietary experiments. As the results in figure V show, this is not the case.

The survival curves establish the following relationships: (a) Both BR and BS mice exposed to simulated high altitude for three weeks are more susceptible to intraperitoneal infection with a suspension containing 250,000 cells of *S. typhimurium* than corresponding control mice regardless of their genetic homogeneity. Moreover, the degree of greater susceptibility of the altitude mice, compared to their controls, is essentially the same for both BS and BR mice. (b) BR control mice are more resistant than BS control mice but the differences are not as great as Webster obtained when he infected the mice by intrastomachal inoculation. This naturally raises the question whether the effect of exposure to altitude on resistance to infection might be better demonstrated by more discriminatory dosages. The experiments described in the section that follows were the direct effort to find an answer to this question.

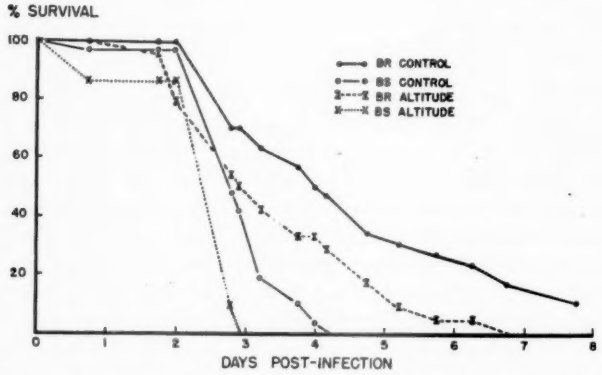

FIG. V. Survival curves of 30 (24 females and 6 males) normal bacterial resistant mice, of 24 (14 females and 10 males) bacterial resistant mice after three weeks at altitude, of 31 normal (27 females and 4 males) bacterial susceptible mice, and of 22 (14 females and 8 males) bacterial susceptible mice after three weeks at altitude. All mice were infected intraperitoneally with 0.5 ml. of a saline suspension of *S. typhimurium* containing about 250,000 cells.

7. *Response of altitude mice and anemic mice to intrastomachal infection with S. typhimurium.* Preliminary experiments established that 50,000 cells of *S. typhimurium* injected into the stomach of normal mice in a total volume of 0.25 ml.

would give a mortality of about 80 per cent within a period of one month. All mice used in this experiment were infected, therefore, in this way. Figure VI presents the survival curves

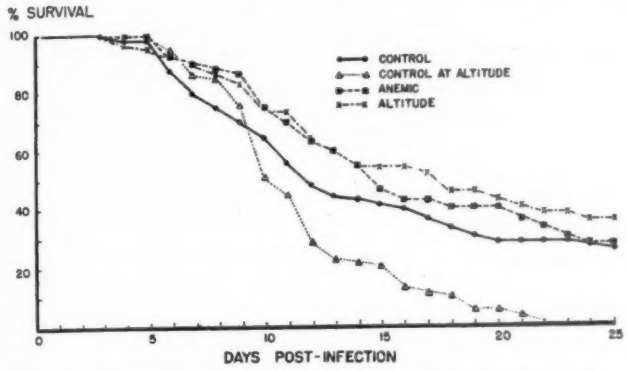

FIG. VI. Survival curves of 60 control mice, of 60 control mice placed at altitude immediately after infection, of 42 altitude mice, and of 60 anemic mice. All were infected intrastomachally with 0.25 ml. of a saline suspension of *S. typhimurium* containing about 50,000 cells.

of the mice in the four groups included in the test. The following relationships are apparent: (a) Normal mice placed in decompression chambers for the entire post-infection period show a greater susceptibility than control mice that remained at normal atmospheric pressures for the entire time after they had been infected. This greater susceptibility is statistically significant to more than the 0.01 level from the twelfth until the twenty-second day when the last mouse in this group died. (b) Mice exposed to simulated high altitude for three weeks prior to infection and then kept at normal pressures for the post-infection period have a smaller mortality than control mice from the second through the third week. This greater apparent resistance is not, however, statistically significant with the number of animals employed. (c) Anemic mice are more resistant than control mice especially during the second week post-infection and the difference is most significant statistically (0.03 level) on the ninth day. However, by the end of the experiment, the survival curve of the anemic mice is almost identical to that of control mice.

Discussion. Mice subjected to a simulated high altitude of 20,000 feet adapt within a period of three weeks by developing a fifty per cent increase in erythrocyte numbers and a proportionate increase in hemoglobin concentration. The sequence of events initiating and accompanying these changes is obscure. In addition to, or because of, the events leading to the altered blood picture, there is the change which manifests itself as a greater susceptibility to infection with *S. typhimurium*. The increase in susceptibility may be small and under some conditions require statistical confirmation but it is real and reproducible. The mortality difference between altitude and control mice is about the same as that observed between Webster's bacterial resistant and bacterial susceptible strains of mice similarly infected. While exposure to altitude lowers the resistance of mice to infection with the organism of mouse typhoid, standardized hemorrhage makes them more resistant to the same organism. The problem now becomes one in which these oppositely oriented changes in resistance to infection are elucidated.

In mice subjected to altitude, neither blockage of the reticuloendothelial system nor immunization against the specific organism alters the greater susceptibility of these animals to intraperitoneal infection with *S. typhimurium* compared to the corresponding control mice. Thus the thirty per cent higher mortality of polycythemic mice seen in figure II may also be seen in figures III and IV. The capacity for antibody production, it must be concluded, is just as great in mice after two weeks at simulated high altitude as it is in mice under normal atmospheric pressures; otherwise, the relative response in the two groups would have been altered. This confirms the direct determination of agglutination titers produced in mice at reduced and at normal pressures. Therefore, the humoral defense mechanism can be involved in the dissimilar response of the two groups of mice only if it is nonspecific in character and is unaltered by stimulation of specific antibody formation. Just as immunization raises equally the absolute defense of the two groups of mice as evidenced by the larger number of bacteria required for infection, so, by similar reasoning, does thorotrast depress equally the defense of these groups. This statement

leads to the inescapable inference that the cellular defense mechanism is not changed in polycythemic mice in such a manner as to increase their susceptibility. Moreover, the data of table 2 show that the spleens of altitude adapted mice take up as much thorium dioxide as the same organs of normal mice. It is evident, therefore, that the greater susceptibility of altitude mice is not to be attributed to an impaired cellular defense, in so far as these results permit a conclusion.

A comparison of the behavior of mice designated as "controls at altitude" with the control group affords additional support for the interpretations presented in the paragraph above. The immune status and the cellular defense in these groups were of necessity identical at the time of infection because normal animals were arbitrarily divided into those that continued to serve as controls and those that were placed in the decompression chambers. The mortality curves prove that during the early days at simulated altitude the mice were, for all practical purposes, identical. This is readily seen in figures III, IV, and V. It was not until the sixth day, figure III, the ninth day figure IV, and the tenth day, figure V, that the mice subjected to altitude became significantly more susceptible to typhoid than controls. The longer time intervals, nine and ten days, were associated either with immune animals or with those infected intrastomachally. When one-half of a group of normal mice infected intraperitoneally with 250,000 bacteria were placed in decompression chambers and the remaining half were kept as controls, there was no significant difference in mortality of the two groups, as seen in figure VII. A tentative timing of the changes in mice at altitude which influence their response to typhoid infection indicates that the first week is a period of essential normalcy while the second week is one in which susceptibility progresses toward a maximum value. Moreover when this change is established, it can then be lost within approximately the same time period when the mice are returned to atmospheric pressures. Thus, the altitude mice that were infected intrastomachally, as seen in figure V, had recovered before the disease developed. Similar curves were obtained with altitude mice infected intraperitoneally one or two weeks after they had been returned to atmospheric pressures (Berry and

Mitchell). In all cases they, like those in figure V, were slightly more resistant than control mice even though statistical significance was not obtained.

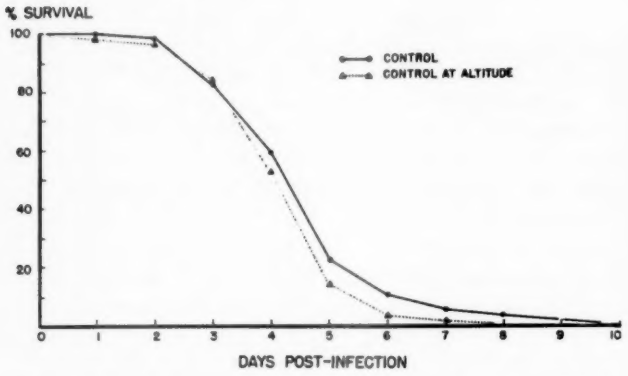

FIG. VII. Survival curves of 57 control mice and of 57 control mice placed at altitude immediately after infection. All mice were infected intraperitoneally with 0.5 ml. of a saline suspension of *S. typhimurium* containing about 250,000 cells.

The mice whose response to infection was reversed by immunization and by blockage of the reticulo-endothelial system were the anemic mice. These animals in each experiment were more susceptible to infection than normal mice, as seen in figures III and IV, compared to the greater resistance seen originally in figure II and again in figure V. In regard to the cellular defense, it should be recalled that earlier experiments clearly established a reproducible and significant increase in the phagocytic activity of blood neutrophiles, of peritoneal macrophages, and in the ability of the spleen to accumulate thorium dioxide from the blood stream, table 2. Thus, if anemic mice are more resistant to mouse typhoid than normal mice, because of the increased activity of the cellular defense, then by rendering this defense inactive with thorotrast injection the greater resistance is lost. But why they are then more susceptible remains to be explained. Since immunization makes the anemic animals more susceptible than immunized controls, it would appear that anemic mice have a lowered capacity for

antibody production. This is contrary to the results of Dreyer and Walker, who found higher antibody titers in animals that had been bled during the period of immunization and it differs from the careful work of Sax from this laboratory which failed to detect differences in antibody production either by determining the agglutination titer or the nitrogen content of a bacterial antigen treated with immune serum from normal and anemic mice. Such contradictory experiments, however, fail to carry the conviction of a proved change in response to infection. Therefore, the results of figure IV must be interpreted as evidence for an impaired antibody response in anemic mice.

Since the stress of altitude alters the susceptibility of mice to salmonellosis in some manner that cannot be explained by demonstrable changes in the cellular or humoral defenses and since Webster's bacterial resistant and bacterial susceptible mice show no differences in the effectiveness of the same defense systems, it must be assumed that these animals respond as they do to infection because of differences yet to be made evident. If stresses elicit hormonal responses and if genetic differences have an underlying basis in enzyme reactions (Horowitz) then there is some justification for postulating a metabolic or biochemical alteration as the mechanism responsible. The recent demonstrations (Berry and Mitchell) that changes in the Krebs tricarboxylic acid cycle influence significantly the course of infection in mice is proof that such factors may play important roles in bacterial diseases. It also shows that susceptibility may be more than the absence of resistance. This is in line with Schneider's postulation which grew out of his work with nutrition and resistance-susceptibility in mice. He suggests that susceptibility is an active mechanism, independent of the defense system as it is classically conceived. To postulate some previously unrecognized mechanism by means of which to alter response to bacterial infection seems necessary in light of these results.

Finally, reference shall be made to those reports closely related to this study. Highman and Altland describe a method for producing bacterial endocarditis in rats. These animals normally are not very susceptible to the streptococci giving rise to such infections but when they are subjected to a simulated

altitude of 25,000 feet for four hours daily for from two to six months, 2 of 3 rats develop endocarditis in contrast to about 1 in 16 controls. In order for this to occur, the bacteria must be injected four times weekly for three weeks immediately after exposure to altitude. Similarly, Harridge and Cole demonstrate that the degree of decompression at 13,000 feet compared to Chicago altitude increases the mortality rate of mice to peritonitis. A strain of *E. coli* was injected as the pathogen. While these experiments were not concerned with the mechanisms responsible, they show that an animal's defense against bacterial infections is impaired by exposure to altitude. It is hoped that additional studies now in progress will add to our inadequate understanding of these phenomena.

Summary

CF–1 mice exposed to simulated high altitude for three weeks and then infected intraperitoneally with *S. typhimurium* after one day of recovery at normal pressures are more susceptible than control mice which in turn are more susceptible than mice made anemic by nine standardized hemorrhages. At the time of infection, the altitude mice have erythrocyte numbers and hemoglobin values approximately fifty per cent above those for normal mice and the anemic mice have values about fifty per cent below normal values.

The thorium dioxide content of spleens of mice from each of the three groups mentioned above was determined by both chemical analysis and by radioactivity assay one day following the intravenous injection of thorotrast. The spleens of altitude mice showed the same activity as the spleens from normal mice while the spleens from anemic mice, which were slightly more than twice the size of normal spleens, took up slightly more than twice the amount of thorium. The average uptake of thorium dioxide as determined chemically was about twenty per cent less than the amount determined by radioactivity.

Altitude mice with the reticulo-endothelial system blocked by intravenous injection of thorotrast were more susceptible to mouse typhoid than control mice similarly treated. This is interpreted to mean that the cellular defense of altitude mice is as

active as that of control mice. Altitude mice immunized against mouse typhoid and subsequently infected with a massive dose of the same organism are more susceptible than control mice immunized and infected in the same way. Changes in the humoral defense of mice at altitude does not account for their greater susceptibility. Anemic mice injected with thorotrast and anemic mice immunized prior to infection are more susceptible than the corresponding control mice. The greater resistance of these mice observed initially can be explained at least partially by the hyperactive cellular defense but this fails to account for their greater susceptibility when this defense is blocked.

Webster's inbred strains of mice selected for their genetic resistance and genetic susceptibility to salmonellosis were each exposed to simulated altitudes in the same manner as the CF–1 mice. The bacterial susceptible mice exposed to altitude are more susceptible to intraperitoneal infection with *S. typhimurium* than normal bacterial susceptible mice and bacterial resistant mice exposed to altitude are more susceptible than normal bacterial resistant mice to the same infection. Since the infectious dose in these experiments was the same as with CF–1 mice, it is important to note that the percentage survival of normal bacterial resistant mice differs from that of normal bacterial susceptible mice by the same amount as for altitude mice and their controls. Moreover, the fact that the mice adapted to altitude in each of the inbred strains are more susceptible than the corresponding control animals of each strain suggests that resistance changes due to altitude are not dependent upon genetic heterogeneity.

Mice infected intrastomachally and then kept in decompression chambers simulating an altitude of 20,000 feet for the entire post-infection period are more susceptible than control mice similarly infected. Mice adapted to altitude prior to infection are more resistant than control mice but this greater resistance is not statistically significant with the number of animals involved. The disease developing after intrastomachal infection appears about one week later and results in deaths over twice as many days as the disease arising from intraperitoneal infection.

These results are discussed in terms of the changes that occur in the defense mechanisms of the animals subjected to stress capable of accounting for the altered response to infection. It is postulated that metabolic differences arise which are responsible for the susceptibility change to mouse typhoid.

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STUDIES OF THE IN VITRO CONVERSION OF CAROTENE TO VITAMIN A*

JOHN G. BIERI AND CLIFFORD J. POLLARD

In the course of numerous experiments on the carotene-vitamin A relationship, Moore (1931) studied the distribution of vitamin A in the tissues of rats which had been depleted of their vitamin A reserve and then fed carotene. He found that the livers of such animals contained relatively large amounts of vitamin A and small amounts of carotene. The vitamin A contents of the lungs, kidneys and intestines were much smaller than those of the liver. The presence of unchanged carotene in the intestine many days after cessation of its administration led him to conclude that the conversion could not take place during the digestive process but must occur in the liver. Olcott and McCann (1931) tested this hypothesis by incubating liver from vitamin A deficient rats with carotene and claimed spectrophotometric evidence for the formation of vitamin A. They proposed the presence of an enzyme, carotenase, in the liver which was responsible for the transformation. Repetition of this work by numerous investigators resulted in both confirmation and denial, with the opposing viewpoints about evenly divided. Wolf and Moore (1932) most effectively criticized the lack of rigorous identification of vitamin A in the work of Olcott and McCann and pointed out the difficulties inherent in such studies. Despite the lack of convincing evidence, during the following fifteen years the opinion became established in textbooks that carotene is converted to vitamin A in the liver by the enzyme carotenase.

In 1941 Popper and Greenberg, using the ultraviolet fluorescence of vitamin A as a means of identification, showed that after feeding carotene to vitamin A-depleted rats, vitamin A appeared within fifteen minutes in the intestinal wall, liver, and various other parts of the body. Matterson (1942), in com-

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* From the Department of Biochemistry and Nutrition, The University of Texas Medical Branch, Galveston. This work was supported in part by a contract between the Office of Naval Research and The University of Texas. Received for publication June 10, 1953.

paring the utilization of carotene when injected into the stomach or into the portal vein of rats, observed that the intestinal absorption of carotene apparently produced a more favorable response than did the introduction of carotene directly into the circulation. The implication that the intestine might be the site of conversion of carotene, inherent in the observations of Popper and Greenberg, and of Matterson, went virtually unnoticed by investigators in this field. In 1946, Sexton, Mehl, and Deuel studied the biological activity of carotene when injected and when given orally, and suggested as a result of their observations that the parenteral carotene was not utilized and that the conversion of carotene to vitamin A might occur in the intestinal wall.

Almost simultaneously Glover, Goodwin, and Morton (1947), Mattson, Mehl, and Deuel (1947), and Thompson, Ganguly, and Kon (1947) presented quantitative evidence from in vivo studies that carotene is transformed into vitamin A by the small intestine, and proposed that this organ was probably more important in this reaction than was the liver. These findings have been substantiated by McCoord and Clausen (1948) and others (see Kon and Thompson, 1951, for a more complete review of this subject).

In vitro studies using rat intestine were reported by Wiese, Mehl, and Deuel (1947) who claimed a significant formation of vitamin A from carotene. In contrast, Glover, Goodwin, and Morton (1948) could not show any definite conversion when carotene was incubated with the small intestine of rats. Using a perfusion technique, Kon and Thompson (1951) were also unable to demonstrate this reaction in vitro. In experiments with calf tissues, Stallcup and Herman (1950) found considerable formation of vitamin A from carotene by both intestine and liver. It should be pointed out that in the two above reports of the successful in vitro conversion of carotene, the analytic procedure for vitamin A employed the reaction with antimony trichloride. This reagent is well known and has been shown to produce colors with substances other than vitamin A. Carotene and its oxidation products yield blue to gray colors. Whereas a correction can be made for the interference by small amounts of carotene, no means exist to allow for the effect of oxidized

carotene. Also, the presence of considerably more carotene than vitamin A in a sample may exaggerate the value obtained for the vitamin. Thus, the validity of vitamin A determinations in which large amounts of carotene and its oxidized products are present is subject to question. Consequently, it must he held that the reports of Wiese *et al.* (1947) and of Stallcup and Herman (1950) cannot be accepted as demonstrating conclusively a significant degree of conversion of carotene to vitamin A in vitro.

More recently, McGillivray (1951) has studied the in vitro reaction of sheep intestine with carotene. Using the incubation procedure of Wiese *et al.* (1947), and estimating the vitamin A by a three point correction of the ultraviolet absorption curve (McGillivray, 1950) he found increases in vitamin A of only fifteen per cent over control segments from sheep on a normal diet. When sheep maintained on a low-carotene hay were used, the increases in vitamin A were considerably greater than in the first experiments. Qualitative proof that vitamin A was present was shown by the reaction of combined extracts, after chromatography, with glycerol dichlorohydrin (Sobel and Werbin, 1945).

Because of the difficulties inherent in analyzing for microgram quantities of vitamin A in biological materials, it is generally recognized that several independent criteria must be used to establish beyond reasonable doubt that a given substance is vitamin A. The criteria employed for this purpose are (1) the absorption spectrum, usually after chromatographic separation, (2) the mixed chromatogram with known vitamin A, (3) the absorption spectrum of the colored product given by antimony trichloride (activated glycerol dichlorohydrin produces a more stable color and is easier to use), and (4) biological activity in curing vitamin A deficient animals. The latter proof is the least conclusive but can be used for verification purposes. Probably the most satisfactory criteria to use routinely are the absorption spectra of the isolated vitamin A and of its reaction product with one of the reagents mentioned above.

The studies reported here were initiated in 1949 after the work of Wiese *et al.* (1947) appeared. It was planned to investigate in detail the in vitro conversion of carotene to vita-

min A by intestine in order to obtain information into the mechanism of the reaction and the possible intermediates involved. Although the study is not completed, the results to date are being presented in the nature of a progress report so that other investigators in this field may perhaps benefit from our experience. The results are largely negative and indicate that the conversion of carotene to vitamin A by intestinal tissue is difficult to demonstrate in vitro, and at best is very inefficient. This raises the possibility that factors not associated with the small intestine may be intimately involved in the transformation of carotene to vitamin A.

Experimental. In all of these studies, commercial crystalline carotene[1] was used. Aqueous solutions containing 20 per cent Tween 40 were prepared as described previously (Bieri, 1951). The general experimental procedure was to remove a portion of the small intestine from the animal as soon as possible after death, flush out the segment with Ringer-Locke solution, fill the lumen with carotene solution, tie off the ends, and incubate in Ringer-Locke solution at 37° C. After incubation, the intestine was drained and in some instances rinsed, and the tissue saponified and extracted in the usual way.

Male rats of the Holtzman strain, weighing 100–175 gm., were fed the U.S.P. vitamin A assay ration for at least four days prior to experiment. Although the animals often were not vitamin A deficient, it has been shown (Mattson *et al.*, 1947), and verified in our laboratory, that in normal rats after several days on a vitamin A-free diet none of the vitamin can be detected in the intestinal wall.

Rabbits of mixed breed and of both sexes, weighing 1–2 kg., were adapted to a purified vitamin A-low diet (Wooley and Sebrell, 1945) for at least four days before the experiment. No detectable vitamin A was present in numerous control segments of intestine.

For the calf intestine, tissue was obtained from a local abattoir as soon as possible after the animals were slaughtered, placed on cracked ice and transported to the laboratory. About 40–60 minutes elapsed from the death of the animal until the intestine was placed in the incubating medium.

[1] Kindly supplied by Barnett Laboratories, Long Beach, California.

Numerous procedures were used to identify and estimate vitamin A present in the intestinal wall. After saponification and extraction with hexane, the dried extracts were analyzed spectrophotometrically and by their reaction with antimony trichloride or glycerol dichlorohydrin (Sobel and Werbin, 1945). The presence of considerable amounts of sterols and of oxidation products of carotene interfered in the determinations and consequently attempts were made to remove these substances. The freezing-out of sterols in dry ice-alcohol mixture was only partially successful, in that not all of the sterols are removed by this technique. Chromatography on aluminum oxide served to separate the excess carotene from the vitamin A and oxidized carotene. A second chromatographing on magnesium oxide-Super Cel (1:1) would occasionally give a sufficient separation of the vitamin A from oxidized carotene so that a vitamin A absorption curve could be obtained with some samples.

Experiments with Rat Tissues. Recovery experiments in which 2.4–12.0 μg. vitamin A alcohol or acetate were added to incubating sections of intestine and carried through the entire procedure, *i.e.*, saponification, extraction, steroid precipitation, chromatography and colorimetric determination, showed that 50–80 per cent of the added vitamin could be recovered. When two deficient rats were stomach tubed with 300 or 500 μg. carotene and sacrificed after five hours, the vitamin A found and positively identified in the intestinal wall was 2.3 and 7.4 μg., respectively. These experiments served to establish that small amounts of vitamin A, *i.e.*, as little as 1 μg., could readily be estimated in the procedure used.

The procedure of Wiese *et al.* (1947) in which intact intestine was incubated 3–6 hours with 300 μg. carotene and the vitamin A in the crude extract of the intestinal wall determined by the antimony trichloride method was repeated with two rats. In each case, a gray-blue, non-fading color developed with the reagent. This could not be considered a vitamin A reaction; when calculated as vitamin A, the amount was less than one microgram. In eight similar experiments, in which the vitamin A fraction of the intestinal extracts was separated from sterols and carotene, conclusive evidence of the formation of

vitamin A was obtained only once, in which 1.4 μg. was found. In about half of the purified extracts, a very flat absorption maximum in the region 325–330 mμ. was discernible, but the colorimetric determination with either antimony trichloride or glycerol dichlorohydrin could not detect any vitamin A.

Similar experiments were performed using minced or homogenized intestine and intestinal mucosa, with negative results. Both aerobic (O_2) and anaerobic (N_2) atmospheres were attempted. Since it has been shown that pyruvate is one of the most stimulatory substrates for intestine (Feldberg, 1943), the addition of this compound to the Ringer-Locke solution was tested; no vitamin A was found. The possibility that adenosine triphosphate (ATP) and reduced coenzyme I (Diphosphopyridine nucleotide, DPN · H) may be necessary in the conversion of carotene was tested with intact and homogenized intestine. No evidence for the formation of vitamin A was obtained.

In other experiments rat pancreatic tissue, commercial pancreatin and bile salts, separately or in combination, were added to intestinal homogenates with carotene, but no conversion was found. Other rat tissues in addition to intestine were tried for their ability to form vitamin A from carotene. These included liver (slices and homogenates) and whole blood. The results with both were negative.

Studies with Calf Intestine. In two separate experiments, 20 mg. carotene in aqueous solution, partially colloidal, was incubated in about 2½ feet of small intestine (50–65 gm. tissue) for eight hours. At the end of this time, the sections of intestine were flushed with saline, and the tissue analyzed. No increase in vitamin A over that found in control sections of intestine was observed. No further experiments of this type were conducted since it was felt that too long a time interval occurred between the death of the animal and the incubation of the tissue. Since it has been shown that flushing of the intestine after incubation may remove some of the mucosa containing vitamin A (Thompson *et al.*, 1950), these two studies are not considered conclusive evidence that calf intestine cannot convert carotene to vitamin A in vitro.

Studies with Rabbit Intestine. Sections of jejunum three to four feet in length (15–25 gm. wet weight) were removed im-

mediately after death by decapitation, flushed with Ringer-Locke solution, filled with an aqueous carotene preparation (3–6 mg. carotene) and incubated 4–6 hours at 37° C. In six experiments in which the contents of the intestine were flushed out after the incubation period, no vitamin A was found in the tissues. From 10–28 μg. carotene were present, indicating that the pro-vitamin had penetrated the gut wall.

In nine additional experiments in which the intestine was simply drained of its contents but not flushed after incubation, evidence for the formation of vitamin A was obtained in seven cases. The chromatographed extracts had absorption maxima at 325–330 mμ. but the curves were very flat. With antimony trichloride, amounts of vitamin A from 0.5–3.8 μg. were indicated; however, the color was always a gray-blue and faded only slightly, if at all. If vitamin A was present in these extracts, the amount was considerably less than that indicated by the color determination. Attempts to apply the correction procedure of McGillivray (1950) to the absorption curves gave values for vitamin A which were two to four times higher than those obtained by the antimony trichloride method. In general, we have found absorption correction methods unsatisfactory for the accurate estimation of vitamin A when applied to such extracts. Inasmuch as control segments of intestine after incubation gave no evidence of vitamin A being present, it is concluded that vitamin A was formed in most of the segments incubated with carotene, but that the amount was of the order of one microgram or less.

Discussion. It is apparent from the results presented that the attempts to convert carotene to vitamin A in vitro have not been very successful. As mentioned earlier, the failure to demonstrate this reaction in rat intestines agrees with the reports of Glover, *et al.* (1948) and of Kon and Thompson (1951). The positive results claimed by Wiese *et al.* (1947) and by Stallcup and Herman (1950) have already been criticized above. Additional comment should be given to the paper by McGillivray (1951) in which sheep intestine was used. This worker was not able to show any appreciable increase in vitamin when carotene was incubated with intestine from sheep on a normal carotene-containing ration. When intestines from

sheep on a carotene-low hay were used, however, increases from 3.7–17.5 micrograms of vitamin A were found. The analytical method used, a spectrophotometric procedure in which a correction is made for irrelevant absorption, was not thoroughly evaluated by recovery or comparison studies in its original presentation (McGillivray, 1950). As mentioned above, this method in our hands has always yielded high values when compared with the antimony trichloride method, even when the latter was felt to give abnormal results due to the presence of oxidized carotene. Consequently, even though McGillivray qualitatively identified the vitamin A produced in his studies, the quantitative aspects would appear to be open to question.

Recently, Rosenberg and Sobel (1952) in abstract form have reported in vitro studies with intestine from normal and diabetic rats, using a procedure similar to that of Wiese *et al.* (1947). These workers used the absorption at 325 mμ. before and after ultraviolet irradiation for the estimation of vitamin A, a procedure shown to be reliable for serum (Bessey *et al.*, 1946). They reported finding significant amounts of vitamin A formed in vitro from carotene, although no values were given. We have tried this procedure with extracts from rabbit intestine incubated with carotene and have obtained "difference" curves resembling those given by vitamin A. In three experiments, the "vitamin A" found was 7.3, 7.0, and 4.0 μg., but on checking the analyses on the remainder of the samples with either antimony trichloride or glycerol dichlorohydrin, no verification of the presence of vitamin A was obtained. Since it has been shown (Bieri and Schultze, 1951) that the irradiation procedure is not reliable in the presence of large amounts of carotene, its validity in experiments of the type dealt with here would seem questionable.

Although there is no doubt that the transformation of carotene into vitamin A in the intact animal occurs in the wall of the small intestine, it must be concluded that the reaction in vitro is exceedingly difficult to demonstrate. Why the system in vitro operates at a very low order of efficiency is puzzling. It has not been proved that the reaction carotene → vitamin A is effected directly by enzymes. If it is enzymatic, the enzymes involved may be extremely sensitive to nonphysiological condi-

tions and thus easily destroyed. Another possibility may be that necessary cofactors are not present in the isolated intestine in sufficient concentration. Perhaps these are supplied by the circulation. The possibility that the reaction involved is a nonspecific oxidation of carotene, one product being vitamin A, cannot be excluded. The ease with which this compound is oxidized and the fact that its oxidation can be coupled with the oxidation of other compounds, make this concept not untenable.

Summary

The results of studies of the in vitro conversion of carotene to vitamin A by rat, calf, and rabbit intestine under a wide variety of experimental conditions indicate that the formation of vitamin A proceeds at a greatly reduced rate, if at all, in the isolated intestine. A review of the recent literature dealing with research in this field is presented.

Added in Press

The paper by De, N. K., and Sundararajan, A. R., The Site of in vivo Conversion of Carotene to Vitamin A. *Ind. J. Med. Res.*, 30: 479–489, 1951, in which they reported unsuccessful attempts to demonstrate the in vitro conversion of carotene by rat intestine, has recently come to our attention.

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VASOCONSTRICTION ACTION OF HYALURONIDASE IN FROGS AND DOGS*

FREDERICK E. EMERY

Hyaluronic acid was first isolated from vitreous humor in 1934 by Meyer and Palmer. Since then many papers and a few reviews (Duran-Reynolds, 1942, 1950; Glick, 1942; Mann and Mann, 1944) of the literature concerning hyaluronidase have been published. These reports abundantly confirm ability of hyaluronidase to facilitate flow of fluid through tissue spaces. Some evidence has been presented (Rigdon, 1940; Brundell, 1942; Elster *et al.*, 1949a; Alyward, 1942; Elster *et al.*, 1949b) to show that the capillary wall may also be made more permeable to dyes. However, Meyer and Ragan (1948) are not in agreement with this and state that intravenous injections of hyaluronidase has no effect on capillary permeability. The present paper is an attempt to clarify this matter by careful measurement of flow from large veins and from lymph spaces in frogs perfused with frog Ringer's solution.

Method. The method of perfusion as used by others (Hooker, 1911; Kurtz and Leake, 1927) was modified so that the minute volume flow could be collected in vials. The amount of the perfusate was weighed in mg. The perfusion cannula was inserted in the aorta at the heart. Perfusion pressure of 36, 24, and 18 cm. of Ringer's solution were used as desired. The composition of this solution was NaCl 0.65 per cent, KCl 0.014 per cent, $CaCl_2$ 0.012 per cent, $NaHCO_3$ 0.02 per cent, and NaH_2PO_4 0.001 per cent. The pH of this fluid was changed as desired over a range 6.65 to 8.00. The addition of hyaluronidase changed the pH very little (see Fig. 1).

One hundred twenty spotted leopard frogs were perfused. In some the heart and large veins were opened and the skin of the body and legs cut to allow free drainage. In other cases the circulation was closed and the fluid drained through a cannula passed up through the ventricle into the auricles. In this way all the venous return to the auricles was collected. In these frogs part of the perfusion fluid drained from lymph spaces through cuts in the skin and the open abdomen into a funnel. Thus both venous and lymph fluids could be collected at the same time. In most preparations perfusion started within 10 minutes after they were pithed but in a few frogs perfusion was delayed in order to study effect of delay on perfusion rate.

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Even after a delay of 30 minutes satisfactory flow was readily established as judged by the amount of perfusate in drops per minute. After a steady rate of flow was established for a period of a few minutes hyaluronidase† was injected into the perfusion tube near the heart. The amount given varied from 1 to 3 cc. of the same Ringer's solution containing 10 to 1,500 turbidity units. The time taken for injection was ½ to 3 minutes; care being taken not to increase pressure in the fluid system.

Results. Figure I-A, represents total flow from open veins and from fluid passing through the vessel walls. After four minutes

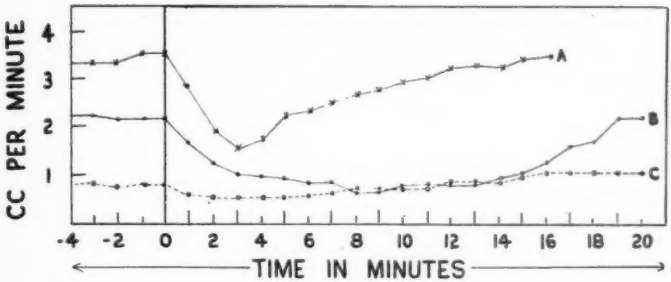

FIG. I. Perfusion volume in cc. per minute. A. Thirty turbidity units of hyaluronidase (Schering) given at the end of the fourth minute. Perfusion pressure 36 cm., body weight before, 28; after, 32 g. Total flow from large veins and lymph spaces. B. Perfusion pressure 24 cm., body weight before, 28; after, 32 g. Flow from large veins. One hundred fifty turbidity units given at end of the fourth minute. C. Lymph flow from wide drainage taken simultaneously with record B. pH of fluid 7.12 before and 7.26 after hyaluronidase (Wyeth).

hyaluronidase† was given and the usual response was a marked reduction in flow. This was followed by recovery to previous rate of flow and sometimes beyond. If the perfusion rate is slow, around 1 to 2 cc. per minute, the effects of hyaluronidase are more marked than was found when the fluid is going through more rapidly. The flow could be regulated by rising or lowering the perfusion pressure.

It has been shown that small vessels in frogs may be cut without fear of loss of fluid as they contract quickly (Herzog, 1925). Careful observation made with the aid of a hand lens

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showed no loss of fluid from cut areas. The amount of perfusion fluid collected as lymph was in some frogs more and in others less than that obtained from all the large veins opening into the auricles.

Volume flow from the veins and from lymph spaces before and after hyaluronidase are shown separately in figure I (B and C).

Discussion. Hyaluronidase has a variable amount of vasoconstrictor action. We have obtained this action with the purest preparation over a three-year period. It is not uncommon to see no effect of hyaluronidase and a few minutes later good vasoconstrictor action by a similar amount given to the same frog. Increased amounts even to large doses did not alter the results to any great extent. This may be taken as evidence that the active agent is an enzyme. Additional evidence that the active agent in these preparations may be an enzyme was evident by the fact that solutions standing at room temperature for a few hours lost activity and in some cases they were completely inactive after standing overnight in the laboratory.

Evidence that the vasoconstrictor substance was not hyaluronidase but some other substance was obtained by boiling hyaluronidase for ten minutes, filtering and testing in the same way on frogs. Very little loss of action occurred. Also hyaluronidase action is greatly retarded by Hesperidin (Sieve, 1952). Frogs were given 1 mgm. of Hesperidin† daily for twenty days and then perfused in the usual way with Ringer's solution to which hyaluronidase in amounts of 750 to 2,000 turbidity units was added. These studies were made in twenty frogs consisting of ten controls and ten injected with Hesperidin. The total flow from the lymph and venous vessels was reduced 39.1 and 42.9 per cent by hyaluronidase in the control and Hesperidin groups, respectively. Thus showing no retardation action of hyaluronidase after Hesperidin.

It seems evident that previous studies which show increased capillary permeability (Rigdon, 1940; Brundell, 1942; Elster *et al.*, 1949a; Alyward, 1942; Elster *et al.*, 1949b) as measured by the spreading of dye could be accomplished after the retarding effect on permeability observed here has worn off. Under

conditions of the present experiments the time would be 10 to 15 minutes after injection.

It is not certain if the reduced perfusion rate observed in the present report was due to vasoconstriction or to swelling of the vessel walls by disturbance in other ways such as edema. The fact that an increase in body weight (average 4 grams) occurred in all perfused frogs, except those injected with Hesperidin, show that tissues were swollen.

Direct observations of blood vessels of the frog's web were not very satisfactory. Slowing and even stoppage of the circulation occurred in some animals after 30 to 750 turbidity units of hyaluronidase were given in the dorsal lymph sac but the walls of the vessels were not observed in the process of constricting.

Temperature ranges from 18 to 40° C. were used. The results did not suggest that temperature was an important factor in these experiments. So most experiments were done at room temperature at about 24 to 28° C.

In 10 dogs anesthetized with nembutal and urethane, hyaluronidase in amounts of 100 to 30,000 turbidity units per kilo was given intravenously in 2 cc. of warm saline solution. The usual result was no effect to a few millimeters rise in blood pressure. Heart and respiratory rates showed small changes consistent with the normal variations seen in these dogs. Evidence of a depressor effect was not noted at any dosage level. It is therefore evident that the vasoconstriction-like action of hyaluronidase seen in frogs and rats (Elster *et al.*, 1949a) is nearly absent in dogs.

Hyaluronidase is not toxic to normal frogs. Their behavior and respiratory rates were unchanged for a period of 24 hours. The amount given was as high as 150 turbidity units per gram injected into the dorsal lymph sac.

Summary

Frogs were perfused with Ringer's solution into the aorta under constant pressure. Hyaluronidase added to the perfusion fluid closed off the volume flow from veins and lymph spaces. Hyaluronidase given in the dorsal lymph sac of frogs some-

times brought about slowing and stoppage of the circulation in the web of the foot.

Frogs injected with Hesperidin did not gain in weight when perfused with salt solution and hyaluronidase, all other frogs showed weight gains.

Following intravenous injections of hyaluronidase blood pressure in dogs rose a few millimeters of mercury. Changes in heart and respiratory rates were within the range of normal. Evidence that the vasoconstrictor substance may or may not be hyaluronidase is discussed.

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THE LEVELS OF X-RADIATION EXPOSURE REQUIRED TO DESTROY AN IMMUNITY TO SALMONELLA TYPHIMURIUM*

JOHN D. FULTON AND ROLAND B. MITCHELL

It has previously been shown that circulating antibodies can be destroyed by exposure to whole body x-radiation (Mitchell *et al.*, 1950, 1951). Further studies showed that it was possible to protect, to a degree, an immune animal's ability to resist a specific post-radiation challenge infection by certain pre-radiation and post-radiation treatment. (Fulton *et al.*, 1951.)

Most of the previous work on this study was accomplished using high levels of whole body x-radiation (450 to 1,000 r). Some of the results obtained were difficult to interpret because considerable numbers of the experimental animals died as a result of the x-radiation alone. The ideal level of x-radiation to use, in studies of this type, would be that level which would destroy the preformed defensive mechanism, developed as a result of immunization, but would not in itself kill any of the experimental animals.

The previous work had also indicated that the challenge infective dose was much too high, because considerable numbers of immunized experimental animals died as a result of the infection alone. The ideal infective dose would be that level of infectious agent (number of virulent organisms) which would not destroy any of the immunized animals but would kill all of the nonimmune animals. Preliminary work has shown the difficulty of attaining this ideal situation. It has been possible to obtain an infectious dose which kills all of the nonimmune animals and about 50 per cent of the immunized animals.

All experimental animals, up to the present time, were immunized by the intraperitoneal administration of a single standard dose of heat-killed *S. typhimurium* antigen. The results indicated that this immunizing procedure was capable of producing good levels of resistance. The animals so immunized were

* From the Department of Microbiology, USAF School of Aviation Medicine, Randolph Field, Texas. Received for publication, May 14, 1953.

far more capable of surviving the challenge infection than were nonimmunized control animals. The immunization dose had not been accurately titrated. It was essential to perform titration experiments to determine if the dose of antigen used conferred maximum protection to the animals or whether a larger quantity should be administered. This information was also essential for the comparison of various batches of antigen as they were prepared.

The work reported in this paper is an attempt to determine at what x-radiation exposure level a preformed specific resistance is destroyed by whole body x-radiation. In addition, attempts were made to further titrate and evaluate the infective and antigenic doses.

Materials and Methods

Animals. The experimental animals used throughout this study were albino male CF-1 strain mice, weighing between 21 and 23 grams. Each individual experimental group throughout the work contained 20 animals.

Immunizing procedure. The animals were immunized by means of a single intraperitoneal injection of 0.5 ml. antigen suspension containing the proper number of heat-killed *S. typhimurium* organisms in physiological saline solution. Throughout this work several different antigen doses were used. The standard dose used previously was 750,000,000 heat-killed organisms suspended in a 0.5 ml. quantity of sterile saline. This was the basic unit of dosage used in this work. The doses used were as follows:

Dose	Number of organisms
X	750,000,000/0.5 ml. normal saline
2X	1,500,000,000/0.5 ml. normal saline
4X	3,000,000,000/0.5 ml. normal saline

Throughout the paper the doses of antigen will be referred to as the (X), (2X), and (4X) immunizing dose. The animals were injected with the immunizing antigen 6 days prior to x-radiation or other treatment.

Infective dose. Several infective doses were used in this work. The standard infective dose used previously consisted of 750,000 live virulent *S. typhimurium* organisms suspended in 0.5 ml. of sterile normal saline (Fulton *et al.*, 1951). It was administered as an intraperitoneal injection. The organisms were cultured and diluted as described in earlier studies (Fulton *et al.*, 1951). The infective doses used in this work were as follows:

Dose	Number of organisms
X	750,000/0.5 ml. normal saline
½X	375,000/0.5 ml. normal saline
¼X	187,500/0.5 ml. normal saline

These infective doses will be referred to as the (X), (½X), or (¼X) infective dose. The infective dose was administered 24 hours post-radiation.

Radiation. The animals were exposed to various levels of x-radiation throughout the study. The x-radiation factors were as follows:

260 KVP, 18 ma.
Filtration of 1.0 mm. aluminum + 0.25 mm. copper.
Filament to target distance (center of animal), 70 cm.
Rate, 55 r/min.

Experimental Procedures and Results. The initial experiment was accomplished to ascertain at what level of x-radiation exposure the ability to resist a specific infection was destroyed.

In this experiment the animals were immunized with an (X) dose of antigen. All animals except the nonimmune infected control group received the immunization. Six days after immunization the animals were exposed to various levels of whole body x-radiation. The x-radiation levels used were 0 (non-x-radiated controls), 50, 100, 150, 200, 250, 300, 350, 400, and 450 r. Twenty-four hours post-radiation, the experimental animals to be infected received an (X) infective dose of *S. typhimurium*. The infection was administered as an intraperitoneal injection. All animals which did not receive the immunization or infection received a control intraperitoneal injection of 0.5 cc. normal saline. Each experimental group contained 20 animals.

The animals were observed for a 15-day post-infection period and the number of deaths in each group were recorded at 12-hour intervals. The results obtained in this experiment are shown in figure I, sections A through I. In these graphs the per cent survival is plotted against time. It is apparent from a study of the graphs that levels of x-radiation below 250 r are not destructive, in themselves, to the animals over a 360-hour (15-day) period. All animals, receiving 50 and 150 r of x-radiation alone, survived the 15-day period. At the 100 r level of x-radiation exposure, 2 animals, which had received x-radiation alone, died during the experiment. One of these died at 192 hours, the other at 204 hours. At the 200 r level, 2 animals, which received x-radiation alone, died during the 12 to 24-hour period. It is believed that these animals and others that died in the early hours of the experiment died from mechanical

injuries or hemorrhages caused by the injection, and not as a result of any of the experimental treatments.

From the 250 r level of x-radiation to the 450 r level, there was a consistent increase in mortalities with each 50 r increment of x-radiation.

Immune animals exposed to 50 r of x-radiation and subsequently infected survived much better than the immune, infected, but non-x-radiated, control animals. This could possibly be indicative of a stimulatory effect of low levels of x-radiation on the immune defensive mechanism of the animals.

The survival picture of immune animals which were radiated and infected was quite similar at the 100 and 150 r levels. Up to 192 hours their survival was almost identical with that shown by immune non-x-radiated infected animals. After 192 hours the x-radiated animals continued to die, whereas the immunized, non-x-radiated, infected animals leveled and their survival picture was unchanged throughout the remaining period of the experiment. At the 360-hour period 30 per cent of the immune, non-x-radiated, infected control animals survived, whereas only 10 and 5 per cent of the 100 and 150 r exposed animals survived for this period.

At the 50, 100, and 150 r levels of x-radiation exposure the immunized, x-radiated, infected animals did not die as rapidly as the nonimmunized, infected control animals. This would indicate that radiation exposure of these intensities impaired but did not completely destroy the animals' ability to combat the challenge infection.

At the higher levels of x-radiation (200 to 450 r) the survival curves of immunized, x-radiated, infected animals were quite similar (in the early hours of the experiment) to that obtained from nonimmune, infected control animals. It is apparent that x-radiations of these levels completely destroyed the animals' ability to resist the infection. In effect, x-radiation at these levels converted immune animals into nonimmune animals. Under the conditions of the experiment the level of

FIG. I. Survival curves of immune animals radiated with various levels of X-radiation and subsequently infected with *S. typhimurium*.

The immune infected control and the noninfected control groups did not receive an X-radiation exposure.

The infective dose of *S. typhimurium* was administered at the 0 hour.

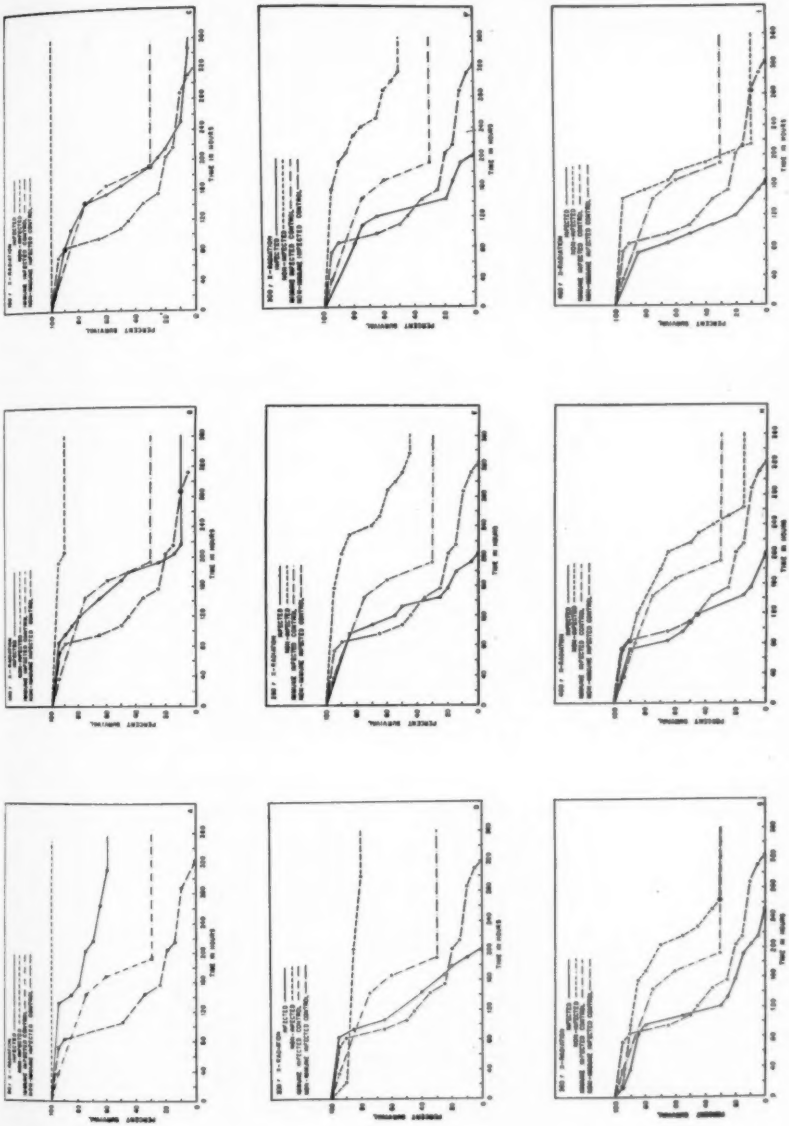

TABLE 1

Time in Hours for the Animals in Experiment I to Reach Mortalities of 25, 50, 75, and 100 per cent.

Per cent Mortality	Non-immune Infected Control	Immune Infected Control	50 r		100 r		150 r		200 r		250 r		300 r		350 r		400 r		450 r	
			NI	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI	I	NI	I
25	92	144	...	204	...	128	...	144	...	102	236	104	240	108	184	98	160	80	160	78
50	108	176	...	...	...	168	...	168	...	130	312	132	312	128	222	110	216	108	186	96
75	156	...	...	...	...	192	...	204	...	168	...	144	...	144	...	132	252	136	204	136
100	324	...	...	...	...	...	....	...	...	204	...	204	...	204	...	252	....	204	...	168
(360 hr.)* Survivors at end of experiment	0/20	6/20	20/20	12/20	18/20	2/20	20/20	1/20	16/20	0/20	9/20	0/20	10/20	0/10	6/20	0/20	3/20	0/20	2/20	0/20

Mortality of this per cent not attained during experimental time period.
NI Not Infected.
I Infected.
* Numerator, number of survivors; denominator, number of animals in group.

x-radiation required to completely destroy the specific immunity as shown by the animals' inability to resist a subsequent challenge infection is between 150 and 200 r exposure. Table 1 shows the time in hours for the various groups of animals in this experiment to reach mortalities of 25, 50, 75, and 100 per cent.

Titration of Immunizing and Infective Dose. Three immunizing doses (X), (2X), and (4X) were titrated against two infective levels (1/2X) and (X).

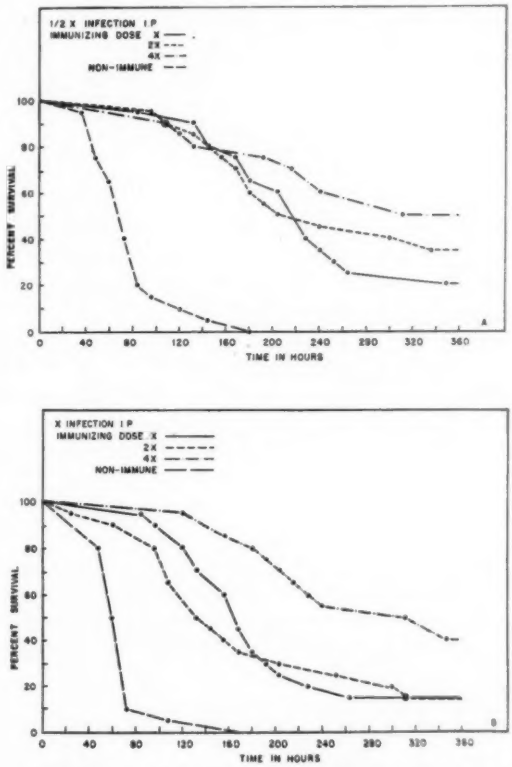

FIG. II. Survival curves of animals immunized with three different levels of antigen and infected with two levels of *S. typhimurium*.
The animals were infected at the 0 hour.

The immunizing dose was administered as an intraperitoneal injection of 0.5 cc. normal saline containing the proper number of heat-killed *S.typhimurium* organisms. The infective dose was administered as an intraperitoneal injection of 0.5 cc. of saline containing the proper number of viable organisms. The infection was administered 6 days after the administration of the antigen. The animals were observed throughout the experiment and the deaths were recorded at 12-hour intervals. Each experimental group contained 20 animals.

The results obtained are shown in figure II, sections A and B, in which per cent survival is plotted against time. The experiment was terminated 15 days after infection.

A study of the graphs shows that each immunizing dose conferred considerable protection to the animals, but the degree of protection increased, as shown by an increase in the number of survivals, with the higher immunizing dose. The protection imparted by a given immunizing dose depended on the size of the challenge dose. The (4X) immunizing dose conferred approximately the same amount of protection to the animals at both levels of infection. Of the immunizing levels tried the (4X) immunizing dose apparently confers the greatest amount of protection to the animals and will be used as the immunizing dose for the remainder of the work.

Table 2 shows the time in hours for the animals immunized with the three levels of antigen and infected with the two levels of infection to reach mortalities of 25, 50, 75, and 100 per cent.

TABLE 2

Time in Hours for the Animals in the Titration Experiment to Reach Mortalities of 25, 50, 75, and 100 Per Cent.

Immunizing Dose	Per cent Mortality of:							
	25		50		75		100	
	½X Inf.	X Inf.	½X Inf.	X Inf.	½X Inf.	X Inf.	½X Inf.	X Inf.
Nonimmune	48	50	68	62	82	68	180	168
X	168	126	215	164	264	204	...	...
2X	156	100	204	132	...	252	...	...
4X	192	192	304	302	...	...	...	...

Inf.—Infection.
Mortality of this per cent not attained during experimental period.

In all but the highest immunizing level the animals receiving the (X) infective dose reached a given mortality much sooner than did those animals infected with the (1/2X) infective dose. The animals receiving the (4X) level of immunization showed the same mortality at both infectious levels. The nonimmune, infected animals (both 1/2X and X infectious levels) reached 25 and 50 per cent mortalities at approximately the same time. The nonimmune animals receiving the (X) infective dose reached 75 and 100 per cent mortalities earlier than did the animals receiving the (1/2X) infective dose.

The work reported here indicates that approximately 200 r of whole body x-radiation in effect converted animals which had been immunized with an (X) dose of antigen into nonimmune animals. It has also shown that larger immunizing doses of antigen protected the animals from a subsequent challenge infection to a greater extent than did smaller doses of antigen. The experiment was repeated to determine the destructive effect of 200 r whole body x-radiation on animals which had been immunized with a (4X) antigenic dose and challenged with three levels of infection. The levels of infection selected were (X, 1/2X, and 1/4X). The animals were immunized 6 days prior to x-radiation and infected 24 hours post radiation. Each group contained 20 animals.

The results obtained are shown in figure III, sections A, B, and C.

In this experiment 200 r of x-radiation destroyed much of the animals' ability to resist the infection but did not completely destroy the specific immunity, as previously observed with radiation exposure at this level. This would not be unexpected as a larger immunizing dosage was administered than was previously used. This larger immunizing dose probably imparted more protection to the animals. All of the animals which received immunization, radiation, and the (X) and (1/2X) infective dose died as a result of the combination radiation and infection, whereas none of the control animals died as a result of x-radiation alone. Ten per cent of the animals immunized, x-radiated, and infected with the (1/4X) infective dose survived. There is, however, comparatively little difference in the form and position of the survival curves ob-

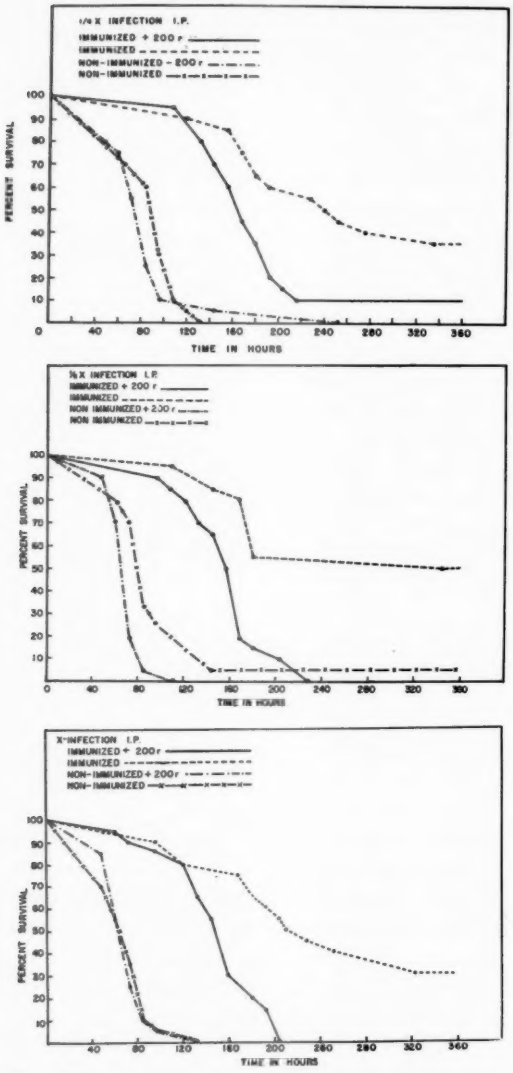

FIG. III. Survival curves of animals immunized with the (4X) antigenic dose and infected with various levels of *S. typhimurium*.

The animals were injected at the 0 hour.

tained from animals radiated and infected with the three levels of infection.

Table 3 shows the time in hours required for the animals in this experiment to reach mortalities of 25, 50, 75, and 100 per cent. A study of the table will show that at each infection level there was little, if any, difference in the ability of the non-immunized, non-x-radiated animals and the nonimmunized, x-radiated animals to resist the infection. The immunized, non-x-radiated animals survived the challenge infection much better than did the immunized, x-radiated animals. The immunized, x-radiated animals attained a given per cent mortality much earlier than did the immunized, non-x-radiated animals. In all cases the immunized, x-radiated and non-x-radiated animals survived much longer than did x-radiated and non-x-radiated nonimmune animals.

TABLE 3

Time in Hours for Animals Immunized with a (1X) Dose of Antigen and Exposed to 200 r of X-radiation to Reach Mortalities of 25, 50, 75, and 100 Per Cent.

Infective Dose	Immune Status	Per cent Mortality of:							
		25		50		75		100	
		XR	NXR	XR	NXR	XR	NXR	XR	NXR
¼X	+	138	168	164	240	188	...	...	...
¼X	—	60	54	74	88	84	98	252	132
½X	+	126	170	156	348	168	...	288	...
½X	—	58	66	65	78	72	108	108	...
X	+	128	174	146	210	170	...	204	...
X	—	54	48	62	64	72	76	132	132

XR—X-radiated
NXR—Non-X-radiated

Discussion. The destructive effect of x-radiation on antibodies and antibody formation is quite well known (Hektoen, 1915, 1918, and 1920; Murphy and Sturm, 1925; White and Dougherty, 1945, 1946; Craddock and Lawrence, 1948; Fischel *et al.*, 1949; and Mitchell *et al.*, 1951).

Taliaferro and Taliaferro (1951) in their excellent review of the effects of x-rays on immunity show that the amount of destruction is dependent upon (1) the quantity of x-radiation administered and (2) the time of administering the x-radiation.

Most of the results of the workers mentioned here were based on the increase or decrease of various antibody titers. As the main interest in this study was in determining the change in the ability of experimental animals to resist or combat a challenge infection, antibody titers were of only slight importance.

Comparatively little work has been accomplished in determining the change in an animal's ability to combat a challenge infection after x-radiation exposure. Gowen and Zelle (1945) showed that x-radiation lowered the resistivity of several strains of mice, each strain having a different degree of natural genetic resistance, to *S. typhimurium* infections. They correlated this x-radiation produced change with the change in the leukocyte number. Zinsser and Casteneda (1932), Liu *et al.* (1941), and Gear and Davis (1942) showed that whole body x-radiation exposure decreased the immunity of rats to rickettsia. Burrows *et al.* (1950) studied the effect of whole body x-radiation on the resistance of guinea pigs to cholera vibrios.

It is apparent that the degree of change in the ability of an experimental animal to resist an infection would depend on (1) the type of experimental animal, (2) the type of infection used, (3) the degree of immunity (both natural and induced), and (4) the level of x-radiation exposure.

Under the conditions in the experiment described herein, the experimental animals (albino male mice) received an immunizing dose of heat-killed *S. typhimurium* organisms. The animals responded to this immunizing dose by producing certain defensive mechanisms (protective antibodies), by means of which they were more capable of withstanding a subsequent challenge dose of live *S. typhimurium* organisms.

When the animals which had received the immunizing dose of antigen were exposed to various levels of 260 KVP — 18 ma. whole body x-radiation, a decrease was observed in their ability to resist the challenge infection. The amount of decrease in defensive ability was proportional to the level of x-radiation exposure up to 200 r. At this level of x-radiation exposure the survival pattern obtained from animals immunized with an (X) dose of antigen, x-radiated and infected, was quite similar to that shown by nonimmunized, non-x-radiated, and infected animals. In effect it seems as though 200 r of 260 KVP — 18 ma.

x-radiation converted immune animals into nonimmune animals by destroying those defensive mechanisms which they had produced in response to the antigen.

Larger immunizing doses (4X dose), apparently stimulated the production of higher levels of protective mechanisms. Higher levels of whole body x-radiation exposures were required to destroy all of the defensive mechanisms. It is estimated that 300 r of 260 KVP — 18 ma. x-radiation would be required to reduce animals immunized with a (4X) dose of antigen and subsequently infected to the status of nonimmune, non-x-radiated, and infected animals.

Summary and Conclusions

Male CF–1 mice were immunized with heat-killed *S. typhimurium* antigen. Six days after immunization the animals were exposed to various levels of whole body 260 KVP — 18 ma. x-radiation. Twenty-four hours after exposure to x-radiation the experimental animals were infected with live *S. typhimurium* organisms.

Under the conditions of the experiment it was observed that whole body x-radiation of 200 r or above completely destroyed the animals' resistance to *S. typhimurium* and in effect converted immune animals into nonimmune animals. The levels of x-radiation required to completely destroy the animals' resistance to *S. typhimurium* is dependent upon the degree of immunity conferred by the immunizing procedure.

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THE LEVELS OF X-RADIATION EXPOSURE REQUIRED TO DESTROY THE ABILITY TO RESPOND TO SALMONELLA TYPHIMURIUM ANTIGEN*

JOHN D. FULTON AND ROLAND B. MITCHELL

Fulton and Mitchell (1952) indicated that whole body x-radiation above 200 r destroys the active immunity produced in experimental animals (mice) by response to a single intraperitoneal injection of heat-killed *Salmonella typhimurium* organisms. In effect, by destroying the animals' immunity to the organisms, x-radiation above the 200 r level converted immune animals into nonimmunes. In other words, immune x-radiated animals could not survive a challenge infection of *S. typhimurium* organisms any better than could nonimmunized, non-x-radiated animals.

The present report concerns an attempt to determine, under the conditions of the experiment, what levels of whole body 260 KVP — 18 ma. x-radiation will destroy an animal's ability to develop an immunity to *S. typhimurium*.

Experimental Procedure. The animals used throughout this work were CF-1 strain, male albino mice, weighing 22 to 25 grams, and were immunized by a single intraperitoneal injection of 0.5 ml. of normal saline containing 3,000,000,000 heat-killed *S. typhimurium* organisms. The animals receiving immunization were given the antigen three days after x-radiation exposure. The animals not receiving the immunizing antigen were given control injections of 0.5 ml. of sterile saline.

The challenge infection was administered as an intraperitoneal injection of 0.5 ml. saline containing 375,000 live *S. typhimurium* organisms. The organisms were cultured and diluted as described in previous reports by Fulton and Mitchell (1951, 1952). The animals receiving the challenge infection were infected six days after receiving the immunizing injection. The animals which were not infected were given a control injection of 0.5 ml. saline.

Groups of experimental animals were exposed, as described previously by Fulton and Mitchell (1952), to the following levels of whole body x-radiation, measured in air with a Victoreen chamber.

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* From the Department of Microbiology, USAF School of Aviation Medicine, Randolph Field, Texas. Received for publication, May 14, 1953.

0 r	300 r
75 r	375 r
150 r	450 r
225 r	

X-radiation factors were as follows:

260 KVP—18 ma.
Filtration of 1.0 mm. aluminum + 0.5 mm. of copper.
Filament to target distance (center of Animal) 70 cm.
Rate 55 r/minute.

TABLE 1

General Schema of Factorial Experiment

Group	Radiated a-a^5	Immunization b	Infection c	Number of animals
O	—	—	—	20
b	—	+	—	20
c	—	—	+	20
bc	—	+	+	20
a	+	—	—	20
ab	+	+	—	20
ac	+	—	+	20
abc	+	+	+	20
a^1	+	—	—	20
a^1b	+	+	—	20
a^1c	+	—	+	20
a^1bc	+	+	+	20
a^2	+	—	—	20
a^2b	+	+	—	20
a^2c	+	—	+	20
a^2bc	+	+	+	20
a^3	+	—	—	20
a^3b	+	+	—	20
a^3c	+	—	+	20
a^3bc	+	+	+	20
a^4	+	—	—	20
a^4b	+	+	—	20
a^4c	+	—	+	20
a^4bc	+	+	+	20
a^5	+	—	—	20
a^5b	+	+	—	20
a^5c	+	—	+	20
a^5bc	+	+	+	20

a-75 r a^3-300 r
a^1-150 r a^4-375 r + Presence of factor.
a^2-255 r a^5-450 r — Absence of factor.

Results

This was a factorially designed experiment with three factors: (1) x-radiation (at a given level), (2) active immunity, and (3) induced infection. The experiment was designed as shown in table 1. Each experimental group (*i.e.*, each treatment combination) contained 20 animals.

The 0 radiation or control groups did not receive any x-radiation but did receive the other combinations of treatment. The animals were observed throughout the experimental period and deaths were recorded at 12-hour intervals. Figure I, sections A through G, shows the survival curves of the animals receiving the various treatment combinations. The individual sections of the figure are comparable and may be studied in relation to each other.

Effect of x-radiation alone. The solid black line in each section of figure I indicates the survival curve of a group of 20 animals exposed to the quantity of x-radiation indicated. These groups of animals received x-radiation (at a given level) only, together with control injections of saline, at the time of immunization and infection of the other groups. A study of the solid line in each section shows that during the experiment (528 hours) whole body x-radiation of 300 r or below was not destructive to the animals. Levels of x-radiation of 375 and 450 r killed 25 and 75 per cent, respectively, of the animals during the experimental period. Exposure to the x-radiation occurred at the zero (0) hour indicated on the graphs.

Effect of immunization alone. As shown by the 0 radiation or control series (Fig. I-A), the immunizing dose (heat-killed *S. typhimurium*) was not toxic to the non-x-radiated animals. None of the animals which received immunization alone died as a result of the antigen injection.

Effect of infection alone. The infective dose of virulent *S. typhimurium* organisms used in the experiment was lethal to the animals. As shown in figure I-A, death occurred in 85 per cent of the animals which received infection only.

Effect of immunization and infection. It was shown above that the infective dose of organisms, used in this experiment, was sufficient to kill 85 per cent of the control nonimmunized

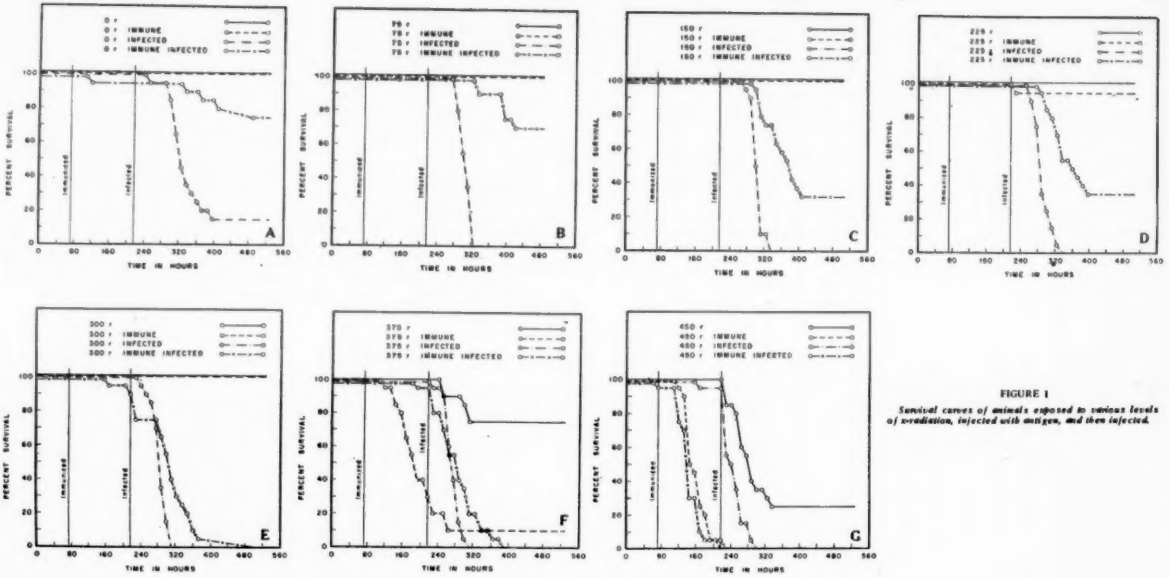

FIGURE 1

Survival curves of animals exposed to various levels of irradiation, injected with antigen, and then infected.

animals. A study of figure I-A shows that while 85 per cent of the infected nonimmune animals died, only 15 per cent of the infected immune animals died. This shows that the immunized animals were much more resistant to the challenge organisms than were the nonimmune control animals.

These results indicate (1) that the infective dose of *S. typhimurium* was lethal for the control animals and (2) that the immunizing procedure was effective in that it increased the animals' ability to combat the subsequent challenge infection.

Effect of x-radiation and immunization. The survival curves of groups of animals which received the combination treatment of a given level of whole body x-radiation and immunization are shown as the broken lines in figure I. For each level of x-radiation exposure there were two groups which received the same combination of x-radiation and immunization. At the time of infection one of these groups received the challenge infection and the other group received a saline injection. Throughout the experimental period none of the animals which received both x-radiation, up to and including 300 r, and the immunizing dose died as a result of the treatment. The one animal which died in the 225 r series died as a result of injuries sustained in a fight. At the 375 and 450 r levels of x-radiation the injection of antigen appeared to have a deleterious effect upon the animals. The combined treatment of 375 r exposure and antigen injection produced death in 70 per cent of the animals within the following six-day period and before the challenge infection was given. A study of figure I-F will show that of the two groups which, up to the time of infection, had received the same treatment (375 r exposure and immunization), one group had 80 per cent deaths whereas the other group showed only 20 per cent deaths during the immunization period.

In the two groups receiving 450 r exposure and immunization (Fig. I-G) all of the animals died before the infection was administered. The obvious discrepancy noted in the 375 r series was not observed in this series. Because of this, it is believed that the adverse effect of combining x-radiation (of these levels) with immunization is real and that the discrepancy noted was due to some unobserved mechanical failure during the x-radiation exposure.

Effect of x-radiation and infection. The survival curves of groups of animals which received the combination of x-radiation and infection are represented by the dash-dot line in figure I.

A comparative study of the effect of the various levels of x-radiation exposure followed by infection indicates that exposures, up to and including 300 r of whole body x-radiation, do not greatly change a nonimmune animal's ability to combat the challenge infection. There is, however, a slight shift to the left in the position of the survival curve at each higher level of x-radiation exposure. This indicates a reduced ability to resist the infection after x-radiation exposure.

It seems probable that the animals have a certain amount of natural immunity (or ability to resist the infection) which is destroyed by exposure to x-radiation. The amount of destruction is directly proportional to the level of x-radiation exposure. This is shown by a comparative study of the survival curves obtained from the non-x-radiated, nonimmune, infected animals (Fig. I-A) and those obtained from nonimmune infected animals which had received various levels of whole body x-radiation. The nonimmune animals which received the combination treatment of 75 r x-radiation exposure and infection tended to die more rapidly after infection than did those nonimmune, non-x-radiated animals which received the challenge infection. It can also be observed that all of the animals which received the combination treatment of 75 r whole body x-radiation and infection died during the experimental period compared with only 85 per cent of the non-x-radiated animals which received the infection but were nonimmunized. As some of the animals that were exposed to 375 and 450 r of x-radiation died as a result of the x-radiation alone, evaluation cannot be made of the effect of radiation, at these levels, combined with infection.

Effect of radiation, immunization, and infection. The survival curves of groups of animals exposed to various levels of x-radiation, then immunized, and then infected are represented in figure I by the dash-x lines. The control animals for this treatment combination did not receive any x-radiation exposure but were immunized and infected. Seventy-five per cent of the

immunized infected animals in the control group survived the treatment compared with only 70 per cent of the animals exposed to 75 r of whole body x-radiation, then immunized, and then infected. Apparently, animals exposed to 75 r whole body x-radiation lost very little if any of their ability to respond to the immunizing procedure.

There is a considerable drop in the percentage of survivors in animals which had been exposed to x-radiation of more than 75 r. There were 30 and 35 per cent survivors, respectively, among those animals exposed to 150 and 225 r. It seems that x-radiation of these levels destroyed much of the animals' ability to respond to the antigen. The animals which received 300 r of whole body x-radiation, immunization, and infection showed a survival pattern quite similar to that of nonimmune infected animals which had been exposed to 300 and 375 r of x-radiation. This would indicate that 300 r of whole body x-radiation destroyed most of the animals' ability to respond to the immunizing antigen.

Animals exposed to 450 r of x-radiation and then immunized died as a result of the combined treatment prior to the time of administering the challenge infective dose.

Discussion. Whole body x-radiation exposure of 75 r (and presumably lower exposures), under the conditions of the experiment, does not appreciably impair the ability of experimental animals to respond to an immunizing dose of antigen (heat-killed *S. typhimurium* organisms). The response of the animals receiving 75 r of x-radiation was quite similar to that shown by the experimental animals which did not receive any x-radiation but did receive the immunizing and infective doses.

A considerable difference in the ability of the animals to respond to the immunizing dose was observed in those animals which were exposed to 150 and 225 r of whole body x-radiation prior to immunization. Animals exposed to 300 r of whole body x-radiation, then immunized, and then infected, exhibited a survival pattern quite similar to that shown by nonimmune animals exposed to 300 r and then infected. Under the conditions of the experiment, 300 r of whole body x-radiation destroyed the ability or capacity of the animals to respond to the immunizing antigen.

The loss of ability to respond to an immunizing antigen after x-radiation has been known for some time. Taliaferro and Taliaferro (1951), in their review of the subject up to 1951, call attention to the following facts: Benjamin and Sluka in 1908 observed lower precipitin titers in animals which had been injected with antigen after receiving several daily doses of x-radiation; Lawen in 1909 otbained similar results, using certain killed microorganisms as antigens. They conclude that doses of X-ray just sublethal to the host unquestionably inhibit the production of antibodies when administered at the beginning of immunization but not when administered much earlier or later.

Numerous workers [Taliaferro and Taliaferro (1951), Hektoen (1915, 1918, 1920), Murphey and Ellis (1914), Murphy and Sturm (1925), and Craddock and Lawrence (1948)] report a destruction of lymphocytes and/or lymphoid tissue by x-radiation exposure. This destruction of lymphoid tissue is accompanied by a correlative decrease in antibody titers. Many workers consider that the lymphocytes are one of the main sites of antibody formation. Any treatment, such as x-radiation exposure, which would destroy the lymphocytes would tend to destroy the ability to produce antibodies. It may be that x-radiation exposure below 225 r partially depleted the lymphoid tissue thereby reducing the x-radiated animals' ability to respond to the antigen. X-radiation exposure of 150 and 225 r destroyed much of the animals' ability to respond to the antigenic dose. Ability to respond to the antigen was completely destroyed by exposure to 300 r of whole body x-radiation. The animals receiving this level of whole body x-radiation, the immunizing antigen, and the infective dose, did not survive in greater numbers than did 300 r x-radiated animals which did not receive the immunizing antigen but did receive the infective dose.

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CREATIVENESS IN MEDICINE*

ALAN GREGG

Well, here you are, after fifteen or twenty years of education, with habits of work and study already formed, with ideas and ideals already established—perhaps, indeed, inveterate—long since accustomed to yourselves and the values and beliefs engendered by your experience. Though change in your ways is still possible, it would scarcely be likely were it not for one priceless characteristic possessed by probably all the higher vetebrates and scientifically shown by Pavlov as the conditioned reflex. That magnificently heartening phenomenon offers us all escape from the life of a foreordained automaton, from habits that deaden, tastes that cloy, and a routine that has gone stale, jejeune, and tiresome. Thanks to that delightful ability to attach a new meaning to an old or hitherto meaningless stimulus, man possesses something akin to rebirth, to the forgiveness of sin, to the opportunity to change and grow, rechanneling old energies in directions quite new.

Still deeper than this encouraging plasticity lies the fundamental fact that so complex and unique is each one of you that I could not expect that any one system of education or any one set of experiences could by now have either stimulated or satisfied all of your potential capacitities or latent desires. As a consequence, you are still ready to begin new quests; you are prepared for a commencement. Mere experience and habit—especially if they have been accompanied by resignation or cynicism—have already, perhaps, determined what you will make out of the next fifty years. That could be the case, but nonetheless there remains the exciting chance that you can change, the lovely chance that tastes and desires that have never yet been quickened in you, or satisfied, can in the future appear for the first time, increase, and finally find their consummation. For that reason I would speak to you on creativeness in medicine—I repeat, for it is perhaps a strange idea, creativeness in medicine.

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* Graduation Address at The University of Texas Medical Branch, Galveston, June 5, 1953.

First, a word or two about creativeness. It has many aspects, but I would call attention to these two: the forerunner of creativeness seems to be the perception of relationships not at first apparent, and then almost in a flash comes the more active phase, the artist's drive to put these freshly perceived relationships into a new arrangement. As an example of such a creative synthesis, the astronomer Halley, passing a graveyard every day, suddenly saw one morning that if he took the ages of all the human beings buried there, he might formulate from such a sample a probable life-span for much larger groups of the human race. The resulting arrangement of the till then unrelated facts on the scattered gravestones led to what we now call life tables. And it is such life tables that have made possible the life insurance business and its unmeasured benefits to man.

As another illustration, when the mathematician Gauss was a little boy, his teacher gave this problem to the class: "What is the sum of 1 plus 2 plus 3 plus 4 plus 5 plus 6 plus 7 plus 8 plus 9 plus 10?" All the other boys began to add the figures given, but little Gauss held up his hand. "What is the matter?" the teacher asked. "I have the answer," said Gauss. "You have heard this problem before!" the teacher said sourly. Gauss replied that he had not. "Then may I have the honor of knowing the answer?" "Fifty-five," said the little boy. "How do you know?" said the teacher, his sarcasm changed to curiosity. "Well, I just noticed," said little Gauss, "that the sum of the first figure and the last figure, 1 and 10, makes 11, and that inside those figures 2 and 9 is 11, and 3 and 8 is 11, and 4 and 7 is 11, and 5 and 6 is 11, so I thought that half the number of terms times the sum of the first and last figures would be the answer." The courage to follow a hunch partakes of greatness.

The perception of relationships and a desire to rearrange or reformulate them—you all will recognize these as cardinal elements in the creativeness of medical research. Let me suggest that in clinical work there is just the same opportunity. The contributions of great clinicians have shown and will continue to prove the wonderful charm of perceiving inherent relationships between phenomena apparently unconnected, and proving the unseen order that some novel rearrangement of them can reveal.

Still another characteristic of creativeness: almost by definition, creation is free—memorably and entrancingly free—to find new fields to play in. Like a parking ticket, orthodoxy must pay a penalty for staying too long in the same place. My time precludes any listing of many possibilities for creativeness in medicine. So I will mention only three, besides the obvious one in medical research. You can bring creativeness, firstly, to teaching, secondly, to arranging for the care of the sick, and, lastly, in that field which above all concerns you—how to create your future self, to convert yourself from being a mere M.D. into being a real doctor.

What we now call the internship system, clinical clerkships, and the full-time teaching of medicine deserved, when they were first thought of, to be called creative ideas. They illustrate creativeness as applied to the working relationships between teachers and students in medical education.

What reason is there for your assuming that the last chapter has been written in the methods and the objectives of teaching medicine? Will your generation have nothing to add, nothing to delete, nothing to change? Is it useless to suppose that there may be a Gauss among you to "just notice" a relationship hitherto ignored that could prove true and enormously rewarding?

Richard Cabot, as much as anyone I know among medical men, created medical social service from his perception of relationships in the hospital care of the sick, which he believed deserved a new arrangement. He played his hunch. I cannot help wondering whether some of you in your day will find in the care of the sick, relationships as yet not recognized, nor rest till you have tried a rearrangement of the factors you have perceived to be involved.

Such creative possibilities may seem remote, abstruse, and impersonal to you, but, as it seems to me, one inescapable issue confronts each one of you and cannot be avoided or evaded: you must create yourselves, you have to apply creation to your own lives as doctors. That you will do, willy-nilly. Probably this means not so much changing your deep convictions, as changing your ways of living so that they will be consistent with your convictions. In that task you just can't help being

personal and original. As Paul Valéry observes, with quiet finality, "Il faut vivre comme on pense: si non, tôt ou tard on finit par penser comme on a vecu." "You must live as you think: if not, sooner or later you'll end by thinking as you have lived."

Creation shares with metabolism a quality of hidden selectivity. It is not just swallowing experience but converting parts of it: not merely acquiring information but achieving a transformation of it. Converting an M.D. into a real doctor is a slow process, for learning calls for getting rid of old attitudes as often as it calls for acquiring new ones. That task will prove sober but exciting; a hidden process but, like successful metabolism, observable to everyone in its results; sometimes a real strain but eventually a triumph.

I can't tell you what to be: you already are. Each one of you, by heredity, by experience, by education, presents, like the patients you will be responsible for, an absolutely unique combination of a myriad of traits and abilities and tastes. All anyone could offer to your peculiar and variant characters will be in essence the raw material from which you'll choose what to take in and to transmute in the individual task of creating yourself. Your future is going to be a state of becoming, not being. When becoming stops, you are dead.

Why do you find this talk on creativeness in medicine so unconventional? I think you would have, as medical students, rather good alibis to the charge of ignoring till now the creativeness of medicine. You began your study of the living—with the dead. Your first patient was a corpse—not much of the spirit of creation there. This introduction was to what had been and long since had ceased becoming. Embryology with its enthralling changefulness may have supplied a brief interlude, but soon began a long apprenticeship to tissue uniformities—of composition, of structure, of function, of response to stimuli, and the grim finality of irreversible reactions. Little of creation there. Description was the tool you plied conscientiously for some two years before narration became a second and less familiar key to unlock the realities of patients' lives. Your thought became centered around repair, recovery, return. You were schooled in humility before the finalities of life. But was

there enough emphasis on life's spontaneities, its adaptabilities, its variability and mutations? So much of given facts, so much memorizing—little that's Dionysian there! Perhaps you found some relief in the parts of preventive medicine and public health that are not concerned with solemnly locking the stable door after the horse—perhaps a Pegasus—has gone.

So I would not wonder if you are surprised at the suggestion that there's much for doctors to reflect upon in the theme of creativeness. Think of Christ's comment on His purpose: "I am come that they might have life and that they might have it more abundantly." There's room for medical creation in those words—"have it more abundantly." If your patients are to have life more abundantly because of your character, then it will pay you to wonder what hinders and what aids your own growth and development as a person.

The chief hindrance to creativeness is the lack of convictions big enough and deep enough to give a lasting pattern to your professional life. Don't plan your future like a pontoon bridge, each petty span no longer than its predecessor—"the vain repetitions that the heathen use." Decide upon fewer arches and bigger ones. Prefer the longer span—the larger symmetry. Keep in mind a symphony; don't be tied to the tiresome reiterations of a popular tune.

The greatest doctors I have known have shown serenity, a serenity that comes only from clear purposes and a firm reliance upon their tenacity to accomplish them. I venture to call your attention to this characteristic of serenity. It comes from a steadiness of conviction, not from frenetic assertions or impassioned credos. There's much in living that cannot be put in words or formulas. Thinking is a part of living: and the whole can never be contained by one of its parts. Learn to avoid overthinking everything you do—but nonetheless remember that unless you have some steady pattern to your existence it will be hard to avoid what Mrs. Wharton neatly calls "getting into the thick of thin things." And strong as your opinions may sometimes be, it may pay you to keep in mind Oliver Cromwell's advice to his zealots: "Gentlemen, I beseech you by the bowels of Christ to remember for just one moment that you may be wrong." Or, as an alternative to so stern a warning, you

may take pleasure in the definition of a zealot as one who redoubles his efforts when he has lost sight of his aim.

Since doctors so often live by patients' confidence, and confidence is the natural child of prestige, the besetting sins of many a doctor become prestige hunting, professional jealousy, and nursing self-pity. Watch out for these, keeping in mind the mellow Chinese proverb that observes "Great men never feel great: small men never feel small." Or, if you prefer advice from Shakespeare, "Ne'er prefer your injury to your heart, lest you bring it into danger."

To counteract the hindrances to creativeness in medicine there are aids to creativeness. I have never known anyone who impressed me as a real person whose world did not seem very real indeed to him. It may be a three-ring circus, or it may be another Golgotha—or anywhere between. But in any case it is a real place, with issues and action that matter. When Carlyle was told that Margaret Fuller had decided to accept the World, he grunted "By Gad, she'd better!" At the bedside of disease, patients do not want and do not need to be attended by mere Quiz Kids—persons as miraculous mentally as they are immature in every other way. That is what I mean when I say there are plenty of M.D.'s in this world but not enough real doctors. In the effort to take the world as a real place and not evade its reality you may on occasion find wisdom in the art of timing, in taking advantage of the fact that life ebbs and flows, that the work of the world, like the work of the heart, goes on by means of diastole as well as systole, that children pass through phases, that enough is enough, that a sense of humor and proportion has its uses as well as being perfectly delightful, and that "good horse sense is the sense that horses have, never to bet on human beings." For you will be let down in the most awkward ways. The elder Walpole had a formula for living that you may find useful: "Good sense, good humor, good manners, and good faith." And there's an antidote for self-pity in Christopher Morley's statement that any man who is worth his salt has by the age of 45 acquired his crown of thorns: the problem is to learn how to wear it over one ear.

But the greatest aid in creating yourself may come from cultivating the art of criticizing yourself. What is the best school

of medicine in the world today? There's a delicate reflection! It could be here, but only by your own resolve, for the best school is that whose graduating class not only knows how to criticize themselves but succeed in holding to themselves the magic mirror of humility incessantly, forever interested in learning. Statistically speaking, the chances of this in any one school are small. But the charm of being an individual is that you don't follow statistics: you make them. The chance may be small, but it is yours.

Learning from whom? Learning from every possible discipline and source. That is the purpose of a university education—to draw from each the best that he can give and, with that rarer grace, to take as well as to give. There is no wisdom in deliberate withdrawal from the thoughts, the companionship, and the company of men who wish to know, to find first principles, to reason, and to share their gains. Osler used to urge doctors to take part in their county medical society meetings for the same reason: to reinforce the urge that all of us have betimes to be as the Passavant Hospital motto says, "More than yesterday, less than tomorrow." Never underestimate your need for the company and the companionship of men who want to learn. And above all have the grace to admit that you can learn. Charles Darwin once observed that in his experience men differed more in the degree to which they used their abilities than in the sum total of their abilities. There lies the path to a creative life: using all that you have and thus getting still more to utilize and glory in.

No school of medicine is worthy of the name that does not teach its students how to learn from experience as well as before experience, how to observe and reason wisely, and to compare their work with what has been observed and thought elsewhere, by others and in other times. He teaches best who shows his students how to learn; not what to think in 1952, but how to think, and how to learn to think, in that long stretch of days awaiting you till, let us say, the year 2000.

All these things are but methods and materials. Yours is, and must be, the creation and the synthesis. It will be yours, nor anyone's so much as yours. With all the methods of diagnosis, the instruments and drugs at your command, now and

in the future, there still will come that crucial moment when you will be the main medicament, the surest comfort, and the brightest hope of every patient, sick or convalescent, who is yours. Don't forget: make today the commencement of the task of creating the doctor you have planned to be and seek forever to improve that plan.

SPONTANEOUS NEOPLASIA IN FEMALE PARABIOTIC RATS*

C. E. Hall, O. Hall, and A. W. B. Cunningham

When rats are placed in parabiosis many of the pairs succumb to a condition which has been termed "parabiotic intoxication." This condition, which usually develops in the second or third week of parabiosis, results in one of the partners becoming small and anemic, whereas the attached parabiont becomes flushed and hyperemic (Dragstedt and Cooper, 1923; Herrmannsdorfer, 1923). Certain of the pairs will manifest the anemic-hyperemic condition, but gradually overcome the condition (Herrmannsdorfer, 1923), a conclusion with which we are in agreement. Increase in the lymphoid tissue, plasma cells and phagocytes, together with hyperplasia of the reticuloendothelial system in the liver of the hyperemic partner suggests the development of a toxic reaction to the enforced partnership (Sauerbruch, 1923; Ernst, 1929). Indeed, it has been suggested that the changes observed in pairs which exhibit intoxication indicate that parabiosis constitutes a form of stress which leads to "adrenal and lymphatic changes similar to those characteristic of Selye's adaptation syndrome" (Diepenhorst and de Vaal, 1950).

Recently it has been observed that in about 40 per cent of the pairs which do not succumb to intoxication, one of the partners develops hypertension, accompanied by widespread cardiovascular lesions, and occasionally arthritis (Hall and Hall, 1951; 1951a, 1951b). This finding was discovered only by maintaining a large series of animals for a period of several weeks, which is longer than such animals have been maintained in the majority of experiments which have employed the technique of parabiosis. Inasmuch as the state of parabiosis leads in many instances to the pathologic changes characteristic of parabiosis intoxication and a more prolonged stress leads to

*From the Carter Physiology Laboratory and the Department of Pathology, The University of Texas Medical Branch, Galveston. This study was supported by a grant from the American Heart Association. Received for publication March 20, 1953.

cardiovascular and joint lesions, it was thought that it might be profitable to maintain animals in parabiosis for an extended period of time in order to determine whether this chronic stress might lead to spontaneous pathologic changes different from those which have been described.

The present communication is concerned with the high incidence of neoplasia which has been encountered in parabiotic female rats which have survived the operation without developing obvious symptoms of either intoxication or hypertension.

Materials and Methods. Over two hundred female rats from our colony, the parents of which were of the Holtzman strain, were placed in parabiosis according to the method of Bunster and Meyer (1933). Many of these either succumbed to parabiosis intoxication, or were used in experiments concerned with the hypertensive syndrome which has been reported elsewhere (Hall and Hall, 1952; 1953). At the time of operation the litter-mate females placed in parabiosis were between 60–100 g. in weight and were paired to within 5 g. in each instance. The diet consisted of Purina Laboratory Chow and tap water fed *ad lib.*

Twenty pairs from the series which were not utilized in any of the above studies, and which had been in parabiosis for periods ranging from 44 to 67 weeks, together with 68 single controls constituted the experimental series from which the data in this communication have been derived.

Results. Pair No. 511. At the age of 11.4 months, having been in parabiosis for a period of 309 days, this pair succumbed. At autopsy many small white ovoid or rounded foci of tissue were found in the organs of both partners. In these rats the tissue was found to have largely replaced the pancreas and to have become disseminated through the peritoneal sac. In the left partner similar nodules were found to be present in the muscles of the thigh and in the region of the thymus. Post-mortem autolysis in the right partner precluded its preservation, although sections of tissue were taken for histology. The gross appearance of the left partner is shown in figure I.

Microscopic examination revealed that the nodules consisted of unorganized masses of rounded and polyhedral cells which varied in size, shape, nuclear content and density of staining. Many of these were multinucleate giant cells, and many of the remainder were found to be in mitosis, both normal and abnormal mitotic figures being encountered. These cells were found to have invaded the pancreas (where normal pancreatic acini could be identified only with difficulty) areolar tissue and skeletal muscle. It was identified as a highly active undifferentiated tumor of uncertain origin, many characteristics of which suggested a carcinoma. Sections from nodules excised from the right parabiont revealed that the tumor in this animal was of identical type. (Figs. II–VI.)

FIG. I. No. 511. Left partner. Note the abundance of tumor nodules in the abdominal cavity. An implant of neoplastic tissue is present in the diaphragm as indicated by arrow.

FIG. II. No. 511. Left partner. Note the tumor tissue invading skeletal muscle on the inner aspect of the thigh.

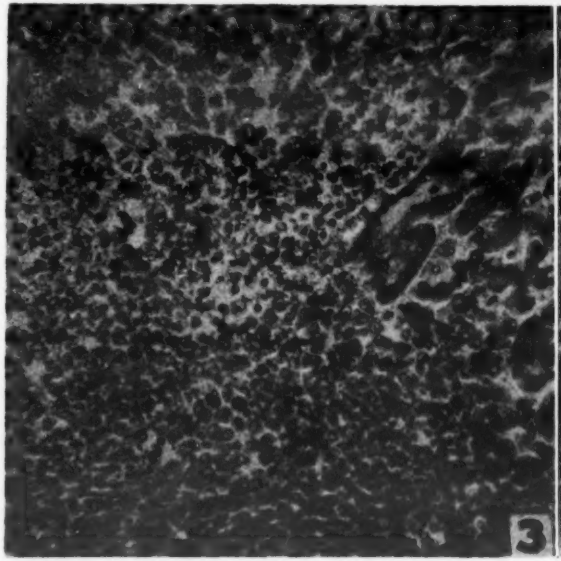

FIG. III. No. 511. Tumor cells invading and largely replacing the cells of the pancreas. Typical pancreatic acini are visible in the upper right-hand corner. (H. and E. X 223).

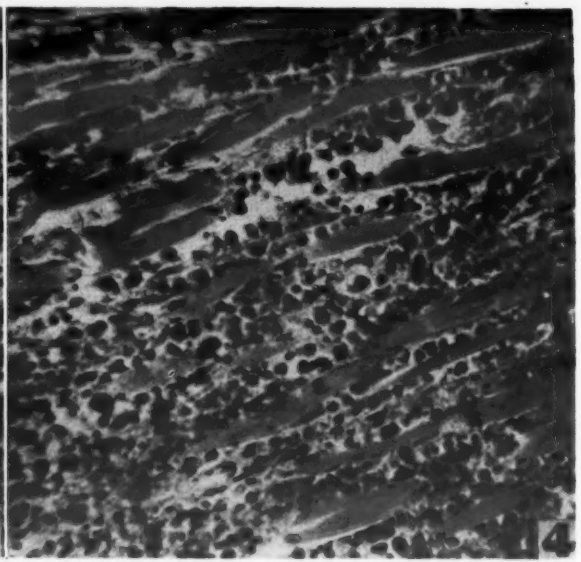

FIG. IV. No. 511. Invasion of skeletal muscle by typical tumor cells. H. and E. X 223).

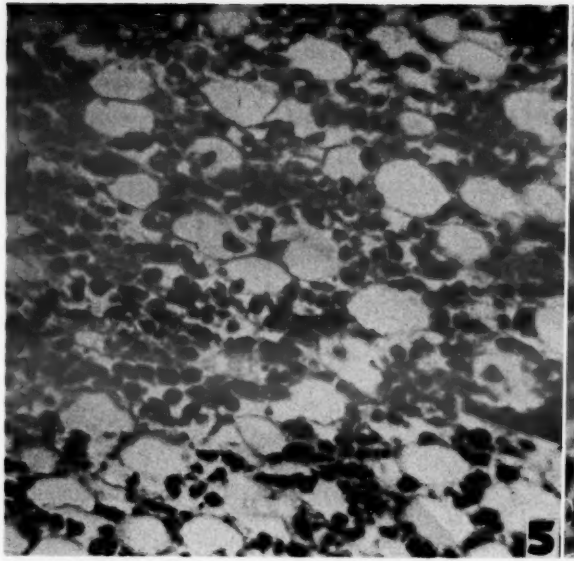

FIG. V. No. 511. Tumor cells invading typical areolar tissue of mesentery. (H. and E. X 223).

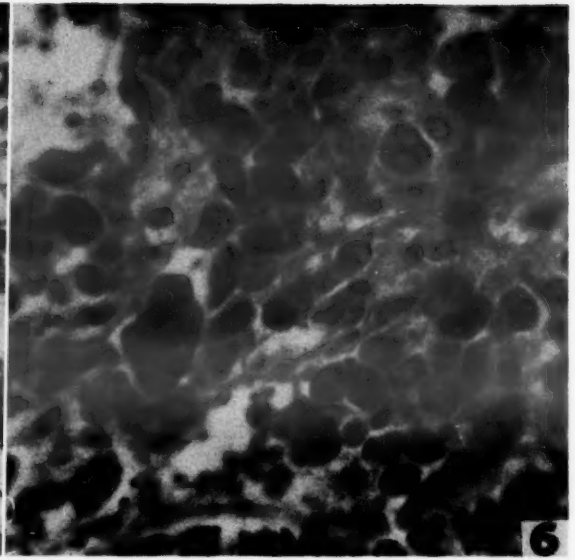

FIG. VI. No. 511. The pleomorphic character of the tumor cells demonstrated: mitotic figures are common. (H. and E. X 741).

Pair No. 521. At 12.3 months of age, having been in parabiosis for 334 days, a large nodular mass was detected by palpation in the abdominal cavity of the right hand partner. The animals were sacrificed and examined. The lymph nodes in the region of the cecum in the right partner had become coalesced together in a confluent hemorrhage and necrotic mass. An isolated hemorrhagic mass of about 1 cm. in diameter was found lying in the lymph nodes around the preputial glands. A small focus of firm, pale tissue was located on the dorsal surface of one of the liver lobes. Sections were taken for microscopic study.

Histologic examination of the involved tissues revealed that the cellular elements consisted of small rounded and larger polyhedral cells: the former possessed a faintly eosinophilic cytoplasm and a single rounded darkly-staining nucleus which, in many instances, was in mitotis. These cells resembled lymphocytes. The larger cells predominated however, and were found to contain large well defined nuclei which were less intensely stained than were those of the small cells. The nuclei were frequently in mitosis and small prominent nucleoli were observed. Multinuclear giant cells were occasionally present. Inflammatory infiltrations into the tracts of tumor tissue were common. Nowhere in the sections was any attempt at cellular organization apparent; rather they tended to form in sheets, subdivided by very occasional collagenous strands. Tumor tissue was noted around the preputial glands, the para-aortic lymph nodes, the cecum, the right kidney, and in the portal tracts of the liver and even hepatic sinusoids. The tumor appeared to be a lymphosarcoma of the undifferentiated reticular type. No tumor tissue was identified in the left partner, however, its right kidney had become a large caseous mass. Microscopically, a granulomatous reaction was present. The structural details of the tumor are shown in figures VII–IX.)

Pair No. 533. During routine examination a nodular mass, which had already reached the size of a walnut, was observed on the mid-ventral surface of the pair on the 251st day of parabiosis. The mass enlarged rapidly and became as large or larger than either of the rats in which it arose. The pair was sacrificed on the 339th day of age (having been in parabiosis for 311 days), for the tumor had become so large that it had begun to show ulceration on its ventral surface from contact with the cage.

At autopsy the mass was found to lie equally on either side of the incisional scar which joined the ventral skin surfaces of the two animals. It was not adherent to the skin, but was well encapsulated. On section it was found to consist of numerous round or ovoid cystic cavities of various sizes. These were filled with a rather thick, viscous, clear, yellowish fluid. The mass weighed 160 g. and its gross appearance is shown in figure X.

Microscopic examination revealed the tumor to be a cystic mass in which secondary infection was a prominent feature. The cytologic structure was quite variable, for where some areas consisted primarily of fibroblastic tissue, in others the stroma had appearances suggestive of cartilage or seemed to have undergone a mucoid change. Many areas of giant cells were encountered. The most common cell appeared as a polyhedral or stellate

structure with ample basophilic cytoplasm and a central nucleus, in which mitosis, always of a regular type, was occasionally observed. The whole tumor was encapsulated by compressed tissues. It was apparently a giant-cell tumor which had arisen either from the periosteum or a tendon sheath. The microscopic features of the neoplasm are shown in figures XI and XII.

FIG. VII. No. 521. Note the large haemorrhagic mass in the caecal region of the left animal (right partner), and the metastatic mass in the region of the preputial glands. The enlarged right kidney of the left partner was due to an infectious process.

Pair No. 527. At routine examination a palpable mass was detected in the abdominal cavity of the left partner. This was first noted on the 391st day of parabiosis when the rats were 14 months old. The pair was immediately sacrificed.

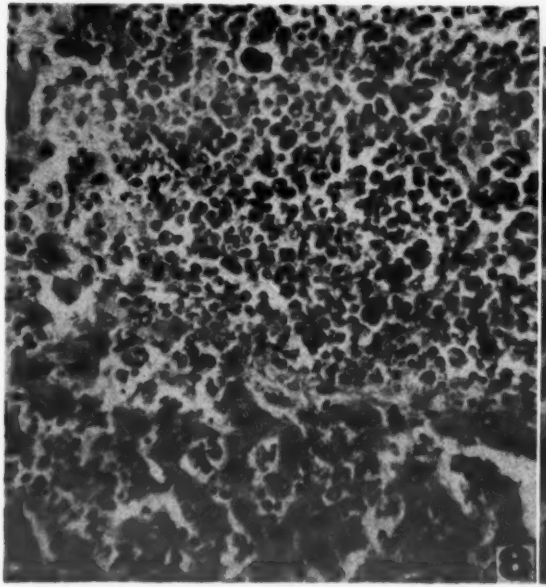

FIG. VIII. No. 521. Tumor tissue invading the liver. Relatively normal hepatic tissue is apparent at the lower right hand corner. (H. and E. X 223).

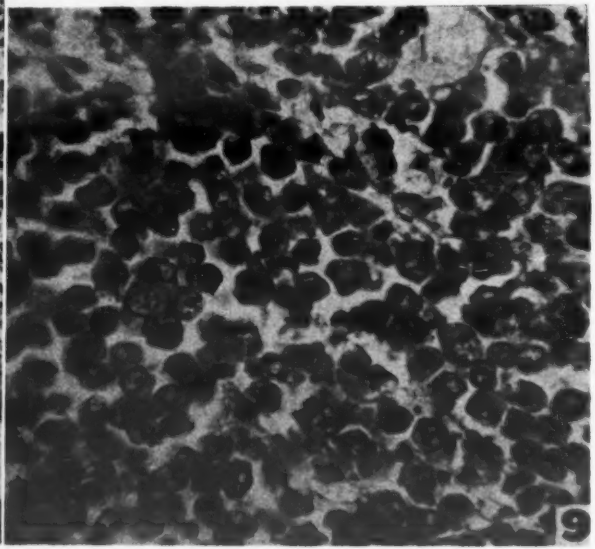

FIG. IX. No. 521. Tumor cells in a lymph node. Numerous mitotic figures are evident. (H. and E. X 741).

Autopsy revealed a picture which, except for the absence of obvious distant metastases, resembled the lesion observed in pair No. 521. The cecal lymph nodes formed a large, confluent, hemorrhagic mass in which necrotic areas were visible. The right partner appeared to be free of tumor tissue.

FIG. X. No. 533. Giant cell tumor of periosteum or tendon sheath in a parabiotic pair of rats. Note cystic nature of the neoplasm.

Microscopically the tissue consisted of a closely packed mass of rounded and irregular cells which varied somewhat in size and shape. Occasional multinucleate giant cells were present. The majority of cells contained large nuclei with a distinct nuclear membrane, fine chromatin material and a fairly prominent nucleolus: the cytoplasm was scanty, granular and

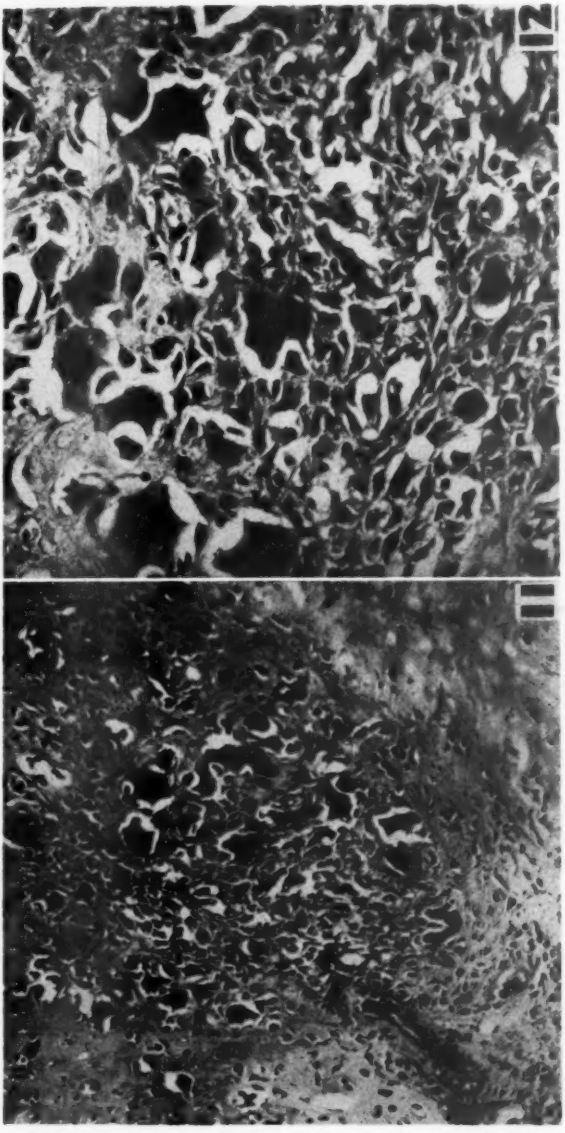

FIG. XI. No. 533. Typical area in tumor showing giant cells. (H. and E. X 223).

FIG. XII. No. 533. Multinuclear giant cells in a typical section area. (H. and E. X 741).

faintly eosinophilic. Mitotic figures were very numerous, about 20 per high-power field being observed. In the involved lymph nodes reticular fibers could not be identified. The tumor was invading its capsule. The structure of the tumor was compatible with a diagnosis of a primitive lymphoblastic type of lymphosarcoma. Metastatic lesions were observed in the lungs, spleen and liver. The gross and microscopic features of the tumor are shown in figures XIII and XIV.

Pair No. 483. On the 441st day of parabiosis the left partner was discovered dead: the right partner, which appeared moribund, was killed. At autopsy the lungs of the left partner were found to contain numerous pyemic abscesses, one of which almost filled the entire thoracic cavity. No tumor tissue was observed. In the right partner the cecal lymph nodes were observed to be markedly enlarged, and a few very small abscesses were observed in the lungs. Tissues from both animals were taken for histologic preparations.

Histologic examination of the cecal lymph nodes in the right partner revealed a massive invasion by tumor cells. These were rounded or polyhedral cells with a rather eosinophilic cytoplasm and a large nucleus with a well marked nucleolus. Mitotic figures were exceedingly common in these. Malignant cells were present in the pulp, sinusoids and in the peripheral sinus. Their abundance in the latter site suggested a spread to the lymph node from some other focus, or a rupture from the pulp into the sinus. No tumor tissue was found in the adrenal, lung, liver or intestinal wall of the tumor bearing animal or in the left partner. The histologic features of the neoplasm, identified as a lymphosarcoma, are shown in figures XV and XVI.

Discussion. Rats subjected to parabiosis are susceptible to three types of spontaneous disease. Acutely many of them develop parabiotic intoxication which, while of uncertain etiology, has been considered due to an antigen-antibody reaction between the partners (Simonsen and Christensen, 1950). Subacutely in many of the pairs one animal will develop hypertension and widespread cardiovascular disease, while its parabiont either becomes hypotensive or remains normotensive (Hall and Hall, 1951). This may well be due to an antigen-antibody response in which the arterioles in the kidney and elsewhere are the victims; an interpretation which is strenthened by the similarity of the cardiovascular lesions to those produced in rabbits rendered hypersensitive to horse-serum (Rich, 1942; Rich and Gregory, 1943).

Chronically, neoplasia occurs with a frequency which renders chance occurrence an unlikely possibility. The incidence

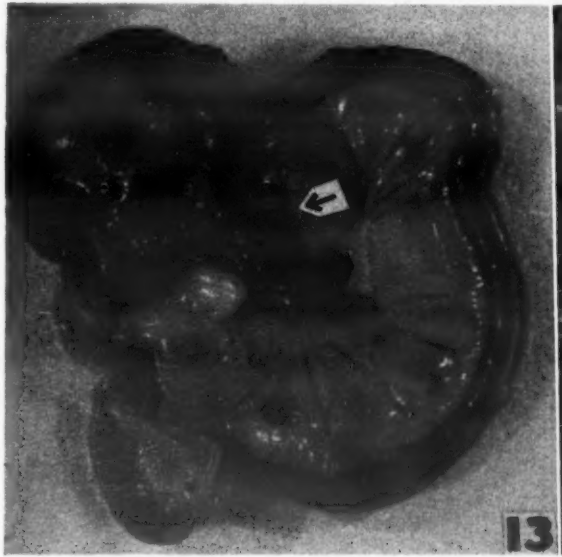

FIG. XIII. No. 527. Intestinal loop, mesentery, caecum and caecal lymph nodes from left partner. Note haemorrhagic appearance of lymph nodes.

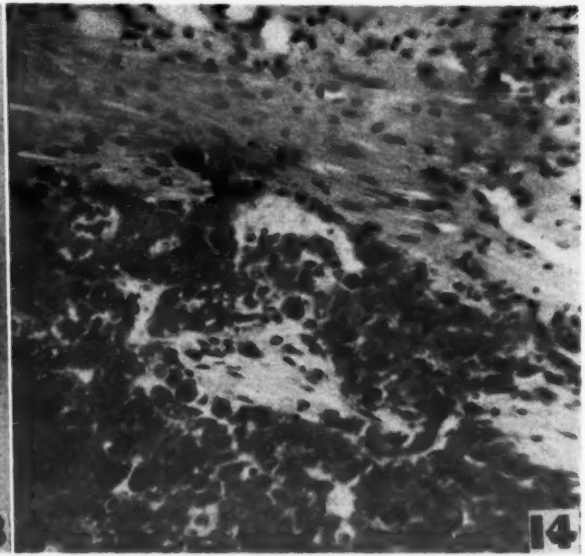

FIG. XIV. No. 527. Same tumor: note pleomorphic character of cells. Mitotic figures are numerous and tumor cells have invaded the capsule. (H. and E. X 223).

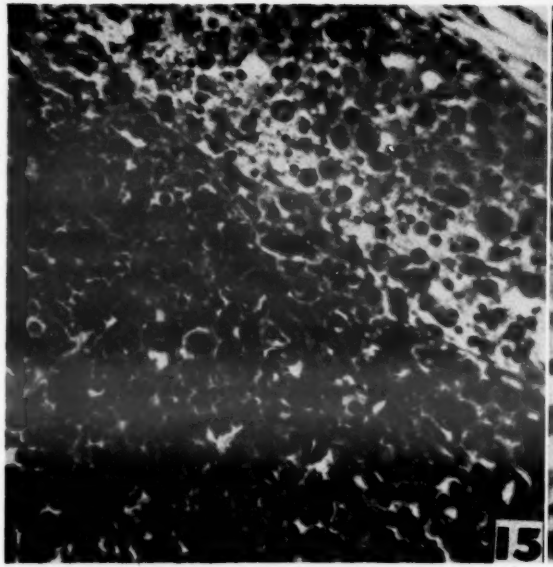

FIG. XV. No. 483. Lymph node from caecal region of right partner. The normal structure is obscured by proliferating neoplastic cells. (H. and E. X 260).

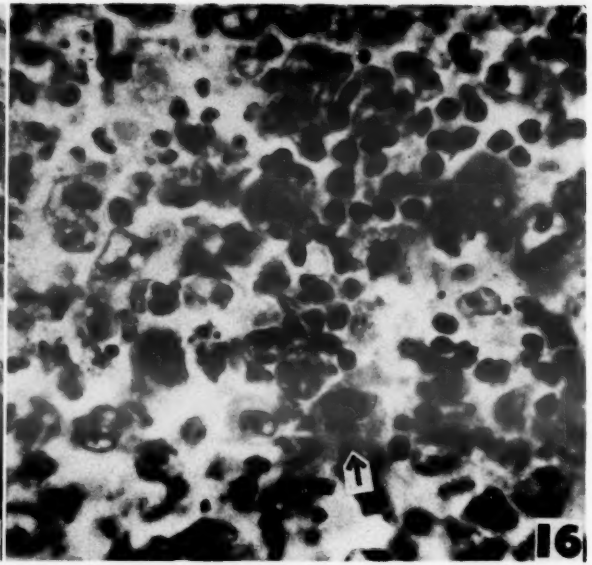

FIG. XVI. No. 483. The same tumor at high magnification. Note variable size of cells and the large mitotic figure in the lower central portion of the photomicrograph. (H. and E. X 741).

at present is five of twenty pairs or 25 per cent; whereas in autopsy of 34 single rats of like age, from the same colony, no evidence of tumor formation has been encountered, nor have spontaneous tumors been observed in any animals from this colony during the four years that it has been maintained. Of the twenty pairs fifteen still survive at the present time, and are being examined periodically for visible or palpable tumors, as are the remaining 34 single animals.

Of the five instances of tumor which have so far been observed in parabiotic rats two have affected both partners, whereas three have apparently been confined to one animal. If the incidence is computed on the basis of pairs, then it has been 25 per cent; however, since the twenty pairs represent forty rats, then the seven out of forty have been affected, an incidence of 18 per cent. In an extensive study of spontaneous tumor formation in 31,868 rats derived from seven different strains, Curtis, Bullock, and Dunning (1931) found that 301 of 12,500 female rats over the minimum tumor age bore tumors of various kinds. The mean "tumor age" of females was 21.8 months. By contrast, in the present instance, the incidence was 18 per cent or 25 per cent, depending upon whether the figure was calculated as the number of animals or the number of pairs involved: the mean age was 12.9 months (in parabiosis an average of 11.8 months), the youngest tumor bearer being 11.3 months of age and the oldest 14.8 months of age. In the series reported by Curtis *et al.*, the tumor incidence in female rats up to 15 months of age was 53 of 9,354 examined, or 0.57 per cent. It is apparent therefore that the incidence herein reported is much greater than chance occurrence would allow. The tumors in our series are of added significance for all of them have been malignant; growing rapidly and in some of them metastasizing, whereas many of those reported by Curtis *et al.* were benign and many were only discovered by a careful and detailed examination of all tissues.

Pituitary tumors have been found to occur spontaneously in intact female rats in parabiosis with castrate partners, presumably as the result of increased estrogen secretion by the ovaries of the intact rats stimulated by excessive gonadotrophin

from the castrate parabiont. In the same circumstances the intact partner may develop ovarian tumors if they are treated with acetylaminofluorine (Bielschowsky and Hall, 1951). Benign or malignant liver tumors and adenocarcinoma of the seminal vesicles often develop in intact males treated with the same carcinogen, when these are in parabiosis with gonadectomized partners, but these do not metastasize to the nontreated partner nor has spontaneous neoplasia been observed (Bielschowsky and Hall, 1951a). Spontaneous tumors, ovarian or otherwise, do not occur in intact mice in parabiosis with castrate partners for periods up to 10 months (Mühlbock, 1952).

In seeking for an explanation for the high incidence of spontaneous tumors in parabiotic rats it is perhaps worthy of note that of the five tumors encountered, three were lymphosarcomas. The lymphatic organs are intimately concerned in reactions to stress, and it may well be that the stress is at least a contributing factor to lymphatic tumor development. Both ourselves and others have noticed that rats placed in parabiosis show early involution of lymphoid tissues. That this may be provocative of later tumor development is suggested by the observations that x-irradiation of mice, a process which produces lymphoid involution, also produces lymphomas (Kaplan, Brown, and Marder, 1951). Similarly the lymphoid tissue of mice treated with keto-hydroxy-estrone at first shows involutional changes followed by hyperplasia and malignant transformation (Dmochowski and Horning, 1952). Further emphasizing the relationship between lymphoid stress and lymphatic tumors is the observation of Willheim and Ivy (1953), that in rats fed various dyes as 4 per cent of the diet, 7 of 33 animals developed lymphosarcoma in a period of 6–20 months, whereas none were observed in fifty control rats. They ascribed the high tumor incidence to "a chronic strain on the lymphatic and reticulo-endothelial system."

While it may be that the neoplasia which results from parabiosis is of quite different etiology from the cardiovascular disease to which many of them succumb, it is worthwhile to consider the possibility, at least, that a common factor is involved.

Summary

In twenty pairs of female parabiotic rats maintained for approximately a year in parabiosis, five pairs have developed spontaneous neoplasms. Three of these were of the lymphosarcoma type and in each case were confined to one partner. In the remaining two cases, a carcinoma and a giant cell tumor of periosteum or tendon sheath respectively, both partners were involved. No tumors were detected in thirty-four control rats. Fifteen of the pairs are still alive and are being observed for tumor formation.

It is concluded that the state of parabiosis predisposes in some as yet undetermined way to tumor formation.

Possible factors which may be involved in the spontaneous tumor genesis in parabiotic rats are discussed.

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THE APPLICATION OF CLINICAL PSYCHOLOGICAL TESTS TO A FULLER UNDERSTANDING OF SOMATIC DISEASE

M. R. HARROWER*

The old adage, *Mens sana in corpore sano*, demonstrates that the importance of psychological factors in medicine was well known to early physicians, while, for philosophers, the relationship of body and mind has always been an intriguing problem. From time to time, as advances are made in both medical and psychological technique, new emphasis and interest will be given to this interrelationship. In the last decades, for instance, the concept of psychosomatic medicine bears witness to this fact.

At the present time a new impetus is given to the interrelation of the psyche and the soma through the development of techniques in clinical psychology which allow of measurement of different components of the psyche, if not in strictly quantitative terms, at least in such a way as to be able to demonstrate relative strengths of demonstrable psychological ingredients. These psychological measuring rods, rough instruments though they still are, permit of a kind of experimentation and evaluation impossible prior to their development. The new rapprochement of clinical psychology and medicine to which both scientific and popular literature bear witness results from the increasing number of problems which become of interest to the two disciplines now that some means of measuring ego strength and impulsive drives are available.

Despite this affiliation of the fields of psychology and medicine, however, the psychologist who would write for or speak to a medical audience faces a dilemma. If he takes time out to make his procedures meaningful,[1] then the results of these

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* Research and Consulting Psychologist, Research Director, Court Intake Project, New York City, Address: 55 East 86th St., New York 28. Presented in part at the New York Academy of Medicine, and at The University of Texas Medical Branch, Galveston, March 28, 1953.

[1] An attempt to make these procedures themselves meaningful to the physician has been undertaken in *Appraising Personality*. (W. W. Norton, N.Y., 1952, 197 pp.)

procedures, the findings, the conclusions, cannot be squeezed into the allotted space or time. However, there is danger in speaking only about the results of psychological tests when the procedures themselves are not understood for such results may appear arbitrary or dogmatic.

Before embarking on the main topic, which will be a consideration of how and in what areas psychological tests have been, and can be, used for a better understanding of the patient as a whole person, it may be well to mention some of the general characteristics which distinguish this clinical type of testing, known as the use of the Projective Techniques, from the concept of psychological tests as it usually exists in the mind of the nonspecialist.

It is safe to say that most persons, at one time or another, have been exposed, in our American culture, to a test of intelligence. In such a test they will have been asked to perform various kinds of mental operations, involving informational knowledge, memory, abstract thinking, arithmetical ability, and the like. Their score—their I.Q.—will depend on the number of correct answers achieved. That is, for any given question, there is one correct answer and one only.

The projective techniques, on the other hand, are characterized by the fact that there is literally no right or wrong answer in itself—the meaning or significance of any one response or answer can be understood only in terms of the total production. These tests are situations, by and large, where the individual is led on to reveal the pattern of his own psychological life, his ways of thinking, acting, feeling and imagining. He is asked, so to speak, to project outwards the picture of the dynamic pressures that are within him.

Lawrence Frank, for example, has said that the projective methods are designed to permit a study of the unique idiomatic individual, that individual being conceived as a process of organizing experience which must elude the investigator who relies on methods that of necessity ignore the patterned quality of each personality.

This projecting outside of ourselves the patterns which are within us is actually nothing recondite or mysterious. The way we tell whether one person is a "tidy" individual or an "un-

tidy" individual is not by looking at his mental processes themselves—but by looking at the room in which he has been living. The "untidy" person has left his mark on the outside world, as has the "tidy" one. We can say a "tidy" or "untidy" person has been here. We never actually know the psychological process of tidiness or untidiness directly. We deduce this from that which has been *projected out, onto the external world.*

The principle underlying the well-known Rorschach Inkblot Test is, of course, just this. What the individual tells us about the inkblots, we know has nothing to do with the inkblots themselves. They have no meaning. In telling us how they look to him, the patient tells us about his own attitudes, feelings and experiences. Looking at an inkblot picture, for example, one seven-year-old twin remarked: "Here are two boys and one boy is *taking* a toy away from the other one." His more passive brother, seeing the same inkblot shortly afterward, described it: "Here are two boys. One is *having his toy taken* away from him." Here identical material is reorganized in two ways—ways which were characteristic of at least *one* aspect of each of the twin's personalities. (Neither answer right or wrong—each is significant only if reinforced by other such answers.)

A couple of analogies may help to epitomize the type of information that is available to the psychologist when the projective material has been assessed and evaluated. We may liken our tabulated results to dials in a powerhouse, indicating various pressures in various parts of a complicated physical system. We are able to tell, even though the individual himself is quite unaware of it, which pressures are, let us say, dangerously near the point of explosion, or, conversely, when there is too little steam to permit adequate functioning. We get a picture of the *hidden* dynamics and the strains and stresses below the surface. If one talks to an individual in a casual fashion, or even examines him in a face to face interview, it may not be possible to get the information about the relative strength of these various strains and stresses. From the projective techniques, we are able to gauge to what extent the individual's psychological energies have been diverted into any one particular psychological system to the exclusion or expense of others. These tests provide us

with ways of assessing to what extent a given disturbance permeates the system at large or is relatively well localized.

Another analogy which may help to epitomize the characteristic of the projective techniques is to visualize footprints in the sand. Seeing these footprints (even if there is no sign of anyone) we can deduce that a man, woman or child, or maybe a dog, walked along ahead of us. In the "meaningless" material, or psychological sand, provided by the projective technique, the individual leaves an imprint of his psychological self and thus, while we cannot see that psychological experience in itself, we are able to deduce some of its properties.

Ironical as it may seem, one could describe a good projective technique as one which appeared meaningless, silly and trite to the participant, for it must give no clue whatsoever as to the type of information it seeks to elicit, and must have sufficiently amorphous properties so that it does not prescribe what should be done with it. Unlike the bottle in *Alice in Wonderland* which said *Drink Me*, the projective material leaves to the subject who is taking the test, the responsibility of deciding what to do with it.

Areas Where Psychological Tests Can Be Used

Let me now outline several different areas in which psychological tests can be utilized in the field of medicine, and then go back to discuss and amplify each area.

(1) They can be used, for example, to assist in the screening of patients for whom plastic surgery may be most helpful. An understanding of the total personality and, in particular the extent to which neurotic difficulties may be related to the physical deformity, may insure better total results. The work of MacGregor and Schaffner may be used to illustrate this particular type of investigation.

(2) Psychological tests may be used for an understanding of the personality type and highlighting of the specific and personal anxieties so that when psychotherapy is utilized as an adjunct to physical measures, it may proceed with a realization of problems at a level not always apparent in the initial psychiatric, or clinical, interviews. Booth's work on the Parkinsonian

personality and his utilization of his findings in therapy would be illustrative of this.

(3) Psychological tests can be utilized to aid in differential diagnosis or as an additional diagnostic instrument in cases where there is doubt in regard to organic cerebral pathology as opposed to neurotic manifestations. Rorschach, Piotrowski, and Harrower, and others, have highlighted the typical "organic" personality as reflected in the Rorschach Test.

(4) Psychological tests can be used for specific research studies as, for example, to measure the effect of some drug on the intelligence level or to assess a change in personality structure. For instance, the work of Zimmerman, Burgomeister, and Putnam on the effect of glutamic acid upon mental functioning in children and adolescents. Or, in the assessing of personality changes following lobotomy.

(5) Psychological tests can be used to validate, correct or modify an initial clinical impression as, for example, in relation to the problem of the psychological effects of ACTH where it has been assumed in some instances that psychotic changes occur in those persons with previously unstable personalities (Barach, Holbrook).

(6) Psychological tests can be used in research projects which attempt to assess the common element in the psychological characteristics of a given disease entity. Or, as a subdivision of the above, to determine whether personality differences can be found when the disease process has a different bodily location. As, for example, cancer of the breast, as opposed to cancer of the cervix, the studies of Tarlau Smalheiser.

(7) Psychological tests may form part of an interdisciplinary approach as, for example, the work of physicians and psychologists at the M. D. Anderson Hospital for Cancer Research, Houston, Texas.

Let me turn now to illustrations in greater detail of each of these areas where psychological tests may be used.

Psychodiagnosis with Reference to Screening

Psychological tests are frequently used for screening purposes, both to "screen in" a particularly desirable personality

type, for some activity, or to "screen out" various undesirables—undesirables, that is, for a particular activity.

One area where screening appears to be of value in the field of medicine is in regard to the selection of persons for the correction of facial defects by plastic surgery. A valuable study by MacGregor and Schaffner in which some of the problems in regard to nasal plastic operations are disclosed, is now being expanded to include the use of the projective tests.

These authors felt that the following criteria, arrived at by the interview technique, might counter-indicate nasal plastic surgery: "(1) Confused, vague, or unconvincing explanations for requesting plastic surgery; (2) Complaint about nasal defect not justified by its actual appearance; (3) Defect which the patient wishes to have corrected is minor, by comparison with a more noticeable physical defect which he apparently ignores; (4) Patient's expectation of results from plastic surgery is excessive or obscure; (5) Outlook for surgical improvement will not meet patient's hopes; (6) Patient's psychologic unreadiness for an operation; (7) Request for surgery due to pressure from family, friends, medical advisors, or others, rather than due to strong desire or conviction on the part of the patient; (8) Patient's history reveals previous nasal plastic operations which consistently failed to satisfy the patient; (9) Patient's history of hypochondriasis, with requests for surgery of various types without adequate cause; (10) Patient's history of consistent, severe maladjustments in life-situations; (11) Placing of responsibility for patient's difficulties in life outside himself, without insight into his own part in them; (12) History of a past psychiatric disturbance, which requires consideration whether the operation may upset the emotional equilibrium. It should be emphasized that wishes to mutilate or remove parts of the body are occasional manifestations in some types of psychosis."

In addition, these authors state, "An experienced psychologic investigator as a member of the team studying candidates for plastic surgery would be equipped to gather and help evaluate the sociologic data pertaining to the patient. In addition he can serve as a liaison between the surgeon and the patient. Through his insights, the patient's problems, anxieties, and expectations can be interpreted to the surgeon, thus enabling him to have a

more thorough understanding of his patient and helping him to be in a better position to foresee or handle difficulties. When interviews and tests reveal deep-seated psychologic disturbance, the surgeon may be forewarned and the patient or his family advised of the need for psychiatric evaluation. The operation can then be held in abeyance until a more definite diagnosis has been made, thereby preventing the possibility of disturbance to patient, surgeon, and hospital and subsequent dissatisfaction on the part of the patient."

The *indirect* approach of the protective techniques often brings to light actual delusional material in regard to a facial defect that may be successfully hidden at interview.

I think of a patient whom I saw recently who commented that the inkblot picture showed a "man with a damaged and distorted nose *like my own*." The patient's nose was in no way distorted. This patient was not a candidate for plastic surgery, but it is safe to say that had he been, he would have been dissatisfied with any result. Since his concern about his nose symbolized a much deeper problem in connection with his concepts of his distorted genitals, a fact which was brought out during psychiatric treatment.

Psychodiagnosis with Reference to Therapy

It has become a common practice amongst psychiatrists and therapists to obtain through the medium of psychological tests as detailed an evaluation as possible of patients who receive psychotherapeutic treatment. Such personality appraisals give the therapists a birdseye view of the individual's strength and weakness and may highlight areas of conflict which the patient, himself, is as yet unaware of or unwilling to bring into the therapeutic session.

It is quite clear that personality difficulties and conflicts are to some extent also a part of a patient who is suffering from a physical disease as well as one whose main trouble relates to neurotic symptoms or emotional disturbances. Many forms of mild psychotherapy must go hand in hand with the treatment of the organic condition. To be aware of characteristics which have shown themselves experimentally to appear more fre-

quently in patients with specific disease entities and to be aware of the peculiar individual problems that any patient is confronted with on a psychological level, must contribute to the overall handling of the patient as a whole. Thus, Booth, as a result of his experimental investigations with psychological tests on Parkinsonian patients, reports that the Parkinsonian personality is characterized "by an urge towards action, expressed through motor activity and through industriousness. Striving for independence, authority and success within a rigid moralistic behavior pattern." As a result of this, he feels "that the therapeutic approach to these patients can be made more successful by respect for their typical personality traits, in particular their excessive shame regarding failures and their need for purposive activity."

The group characteristics of patients with various physical diseases are, as we shall see later, interesting areas for research studies. But the diagnostic appraisal used therapeutically is more valuable when concerned with the person as an individual, rather than as a member of a group. To point this up, let me quote two cases where, with the identical symptom of headache, the detailed psychological evaluation highlighted two very different personality problems.

The first patient, Mrs. A., was described by her physician as, "A young woman of 21, who gave a history of frequent disabling migraine headaches which had first appeared 11 years before and which had become worse in the past two years. They were of the typical migraine type, one-sided, throbbing, and accompanied by nausea and lassitude. They usually lasted three to five days. Partial and intermittent relief was obtained by the use of codeine. The headaches were prone to occur before important engagements. They were unrelated to specific phases of the menstrual cycle."

This patient, on detailed psychological examination, proved to be an unusually intelligent girl, with a personality picture which, at the moment, can be described as well within normal limits. However, three important areas of conflict stood out very clearly. Undue perfectionist tendencies, rigid barriers against the expression of any aggression or hostility and a sustained struggle and conflict over the legitimacy of her exhibitionistic desires. In this instance, the physician who was treating her, combined medication with a frank discussion of her problems as they had been reflected in the tests. This patient was able to utilize these insights and has since reported fewer headaches and that, more important, when they occur, she has been able to relate them to specific situations where tenseness, as she describes it,

became "a rising cumulative process." This tenseness, she realized, followed frustrations and conflict situations in which she somehow maneuvered herself and which she then could neither resolve nor handle.

Patient B., a 17-year old boy, who was referred initially because of his complaints of headaches, presented an entirely different problem when envisaged through the test media. In terms of the total picture, the headaches were incidental. The test revealed that this boy was profoundly disturbed psychologically, appearing as a borderline schizophrenic. Extremely cautious psychiatric care was necessary and, in this particular instance, a period of hospitalization was ultimately resorted to. Test findings here bore no relationship to the somatic symptom of headache. And the psychological recommendations, as a result of them, pointed in turn to the general profoundly disturbed psychological picture, and could give no help in regard to the symptom itself.

Another example may be given which reflects not so much suggestions from the psychological testing as to the choice of therapy as an indication to the physician in regard to his general orientation and attitude towards the patient. Let us contrast in the accompanying chart two patients, each of whom display in the interview with the physician and in the test-situation with a clinical psychologist what might be described as "overtly distressed" behavior. A systematic battery of tests, however, reveals that while these two patients reveal superficially similar behaviors, their underlying personality structure, their capacity for growth, their intelligence, their stability, differs markedly. One patient will be shown to be a basically poor risk while the other, despite momentarily being "thrown off balance," is essentially a stable, intelligent and creative individual. Such a brief epitomizing of test findings may be used to assess the personality characteristics of any given patient.[2]

Summary of Test Findings

Or, in terms of a research design, they can be used to summarize the findings from large groups of individuals. In estimating, let us say, the effect of certain procedures on two selected groups of patients, their initial psychological status can be appraised in this manner and contrasted after a time interval in which the procedure in question had been tried.

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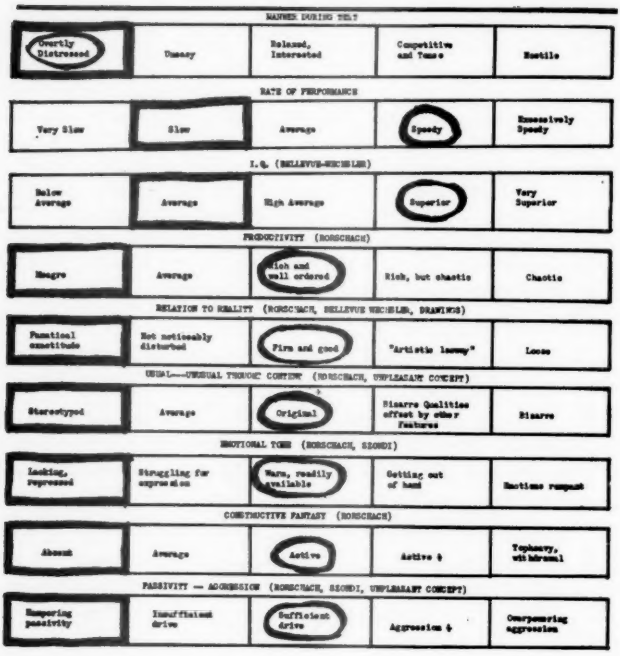
[2] A detailed description of this summary chart is available in *Appraising Personality*. (W. W. Norton, N.Y., 1952, 197 pp.)



Psychodiagnosis with Reference to Differential Diagnosis

The use of psychological tests in differentiating between the patient with organic cerebral pathology and the patient with neurotic symptoms simulating a cerebral lesion, was one of the earliest uses made of the projective methods in clinical medicine. The so-called "organic personality" as reflected in the tests, is perhaps the only one which may be spoken of as a bona fide psychological entity. There is a uniformity in the personality profiles of the organic patient which is quite startling. This uniformity results it would seem, from the dropping out, or masking of the many differentiated aspects of the personality so that, regardless of the wide varieties of personalities in whom brain tumor may occur, a uniform, stereotyped, constricted and impoverished personality results as a psychological concomitant

of the cerebral pathology. This is easily demonstrated by a graphic portrayal of the test findings.

The Rorschach Test, the instrument which measures this particular "blacking out" of individual personality characteristics most effectively, can also be utilized following removal of tumor to trace the expansion of the personality and its increased differentiation over a period of time. Studies which have followed patients for as much as three years after operation will show quantitatively the reappearance of various differentiated forms of reaction which had previously been submerged or lost. Individuals who show this characteristic blacked-out or stereotyped personality profile may still retain high intelligence, and examination by intelligence tests alone may reveal no defects whatsoever (Harrower-Erickson).

A variation on this diagnostic theme is seen in a case reported by Pasquarelli and Bellak where the question of "either idiopathic epilepsy and hysterical convulsive seizures," was restated in terms of the demonstrable coexistence of two distinct personality patterns.

Psychodiagnosis as a Measuring Rod of Improvement

We mentioned earlier the study of the effect of glutamic acid upon mental functioning of children and adolescents as an excellent example of how quantitative measures can be introduced through the tests to substantiate and elaborate the clinical impression. In this experiment by Zimmerman, Burgomeister, and Putnam, 69 patients were examined, 28 were children with convulsive disorders, 11 of whom are mentally retarded also. Thirty-three of the 69 were mentally retarded without convulsions. The Stanford Binet Intelligence Test, the Bellevue-Wechsler Intelligence Test and the Rorschach Inkblot Test were used to measure changes in intelligence and personality structure, respectively.

Following a six-month period of treatment, the I.Q. (Intelligence Quotient) of the retarded group rose from five years eight months to six years eight months, or a year's growth in mental age over a six-month period of treatment. This is twice as fast as would occur among untreated children of average intelligence

since an average gain of six months in mental age accompanies a change of six months in chronological age. For the entire experimental group, there was a gain of 13 months—six years ten months to seven years eleven months during a six-month treatment period. Individual patients showed improvements as great as 13 points on the weighted scale of the Bellevue-Wechsler, rising, for example, from 107 to 120, or changing from the Average to the Superior group of the total population.

As far as the personality tests were concerned, the initial Rorschach records of these subjects were extremely stereotyped and meager. Their performances were characteristically unproductive. Their relation to reality was extremely poor. Following glutamic acid, consistent improvement was revealed both in productiveness and accuracy. A greater facility for the perception of details was apparent and responses indicating better *social and emotional* adjustment were also found in the post-treatment records.

The authors feel that glutamic acid has an effect on mental functioning which is measurable not only by intelligence performance tests, but which reflects itself in basic personality changes. Glutamic acid, they conclude, on the basis of these quantitative changes, accelerates mental functioning in human subjects and this facilitation is found in verbal, motor and imaginative levels. The most striking changes appeared in the most seriously retarded group where statistically significant differences are obtained between test and re-test intelligence quotients. In many cases, a greater degree of emotional stability resulted.

Psychodiagnosis with Reference to Hypotheses

A battery of psychological tests can be used to prove or disprove hypotheses with regard to personality changes following the administration of ACTH or cortisone. For example, there is, at the present time, the suggestion based on clinical impressions that the persons with unstable personality structures initially—are those in whom the drug precipitates a psychotic episode. Although insufficient evidence is available at the present time, it would appear, from the psychological tests, that

such a hypothesis is not tenable. Among those in whom acute psychotic episodes have been precipitated are persons with test findings well within normal limits prior to the administration of the drug.

On the other hand, other studies with ACTH suggest parallel psychological and clinical findings. Barach has recently reported that asthmatics treated with ACTH experience what he describes as "a heightened awareness of life." The assessment of *test findings* of patients on ACTH for arthritis—obtained by one investigation and assessed, independently, by another (Holbrook)—has demonstrated quantitative changes occurring in the Rorschach such that there is a rise in those scores relating to the capacity to experience and express emotional responsiveness more fully. This could well be the test parallel to Barach's clinical observations. Large-scale investigations of this kind, under carefully controlled conditions, might lead to a better understanding of the action of this drug.

Psychodiagnosis with Reference to Personality Types in Different Diseases

Reports of attempts to find quantitative test evidence of "typical personalities" in various diseases may now be discussed. From many examples available, a few, which are typical, may be considered.

Rosacea or Morbid Blushing. An experimental study reported from England by Plesch relates to the psychological examination of 50 patients suffering from rosacea and/or morbid blushing and 50 suitable controls. The so-called neurotic signs of Harrower were taken as a rule-of-thumb measurement for psychiatric morbidity. It was found that five or more of these signs (there are nine in all) were seen in 78 per cent of the experimental group and only 20 per cent of the controls.

Particularly characteristic of the personality disturbance of this patient-group was an undeveloped or faulty ego structure and poor emotional and impulse control. The author, however, points out that this does not appear to be characteristic of rosacea or morbid blushing alone, but has parallels in other psychosomatic illnesses. He quotes, for example, the work of

Brown on duodenal ulcer and Scatia on asthma. Plesch also finds, however, from the test scores that there is a strong paranoid tendency in rosacea, paranoid signs being present in one-quarter of the patients protocols and compared with one-tenth of the controls.

Duodenal Ulcer. Two studies may be quoted as representing work which has attempted to elicit personality traits that are typical of the peptic ulcer patient. A study by Osborne and Sanders, using particularly the Rorschach method, finds "several fairly clear-cut Rorschach patterns discernible. The typical patient in the group studies is characterized by emotional immaturity reflected by his impulsive and instant response to his feelings. Demands for food, love and attention and protection must be satisfied immediately. The primitive instincts of the child are in control. These patients are likely to respond to life-situations as the infant would who does what he pleases without regard to time and place. On the other hand, they also find an unrealistic ambition and an intellectual drive which is far beyond the subject's creative ability. These patients, then, seem to behave on the emotional level as children while intellectually showing strong self-assertive and ambitious tendencies which are not in proportion to their creative capacities.

In an independent study, Malcolm Brown *et al.*, claim that the Rorschach technique provides a method whereby the dynamic organization of personality structure can be quantitatively evaluated on the basis of the individual's responses to the test. Their studies dealt with 25 male war veterans who were receiving treatment for duodenal ulcer and 25 patients with various complaints, none of which could be referred to the gastro-intestinal tract.

Their observations show statistically significant differences in the records of the two groups under consideration. They felt that ulcer patients as a group tend to deal with their environment in an impulsive, emotional, and immature level, leaning to conflict in the area of social, interpersonal relations. This is closely allied to the study of Osborne, and the raw data from which these conclusions are drawn also parallel Osborne's findings.

Multiple Sclerosis. Harrower and Kraus recently administered a battery of psychological tests to 140 patients with multiple sclerosis and a comparable battery of tests to five independent groups of controls. These controls included 200 patients with emotional disturbances, 258 "normal" individuals or persons without overt psychological or somatic disturbances, 29 Parkinson patients, 21 polio patients. This study followed on a previous pilot study in which we had found that while no typical, stereotyped or uniform personality picture of the patients with multiple sclerosis emerged (such as had been found by investigators in cases of brain damage) nonetheless, in every test used, there were some items which differentiated the multiple sclerotic group from the controls. These findings, in general terms, could be epitomized as showing in the multiple sclerotic patients an overemphasis on dependency needs, a virtually complete absence of body-centered anxiety, a minimum of inner conflict or an attitude of resignation and unrealistic tendency to see the world as through colored glasses. We felt that it was important to emphasize that the discovery of certain trends in the group as a whole did not mean that every patient in the multiple sclerotic group showed these characteristics. It was only that the incidence of these traits was significantly higher in this group than in the control group. Individual multiple sclerotic patients deviated markedly from the general pattern, some giving no indications of the characteristic constellation of the findings.

Our large-scale study reinforced the findings of the initial one. Those same traits, and particularly those of over-dependency and an unrealistic absence of body-centered anxiety were as strongly evidenced as before. A breakdown of our multiple sclerotic group into cases during remission, cases which were mild and cases which were advanced, showed certain distinct group characteristics.

Characteristic of the patients in remission was a higher percentage of persons with superior intelligence, the lowest amount of erraticness of performance. We also found that, on the personality test, they showed a capacity for richer psychological experience in living. There was the highest percentage of expanded personality types; a further characteristic could be

described as a more realistic willingness to allow the anxiety component to register, as such, in consciousness. Here one could raise the perplexing question: Do these so-called better personality characteristics allow for remissions to take place, or are they the result of changes in the disease process?

Among the patients with mild, early forms of the disease, were found the greatest amount of overt distress as regards the experience of the illness itself. One could say that the amount of anxiety related to the disease was in inverse proportion to the extent to which the patient was seriously handicapped at the time of the examination.

We also found that in the specific environment of the Kaiser-Kabat Institute where MS patients were encouraged to be as independent as possible, that the so-called dependency scores were higher among those patients examined immediately on arrival than in those who had been exposed to the "treatment." These patients also showed quite a shift towards extrovertial living as reflected in the Rorschach Test.

As a result of these findings, we were left with a feeling that the psychological characteristics could not be considered as predisposing the person to the disease. Rather that the disease imposed certain standard conditions on the individual patient leaving its mark to a greater or lesser degree, depending on the patient's pre-morbid personality structure. When living conditions were changed, changes were reflected in the test score.

Hypertension. Booth has contrasted the personality profiles of Parkinsonian patients with those with *hypertension*, finding certain "group" differences quantitatively demonstrable from the test findings. While the Parkinsonian patients are dominated by an urge for individualistic independent action, obstacles being liable to provoke aggressiveness in excess of the chances for success, he finds that patients suffering from hypertension have a tendency toward dependent relationships in forms of identification with their social environment. Action is determined by material needs and by social standards. Obstacles easily provoke conflicts between dependent and aggressive impulses resulting in restriction of the range of action and of perception.

Hypertensives have also figured in a study by Modell and Potter in which the drawing of the human figure was utilized in the testing situation. Hypertensives, peptic ulcer and asthmatic patients were contrasted. These authors find that some psychopathic features seen in the drawings, occurred in *all three disorders* to about the same extent. This was especially true of the graphic evidences of disturbances in psychosexual organization and withdrawing elements indicative of wariness and suspicion of the environments. *However, there were also characteristics which pertained exclusively to each group.*

For example, in the hypertensive group, inner contradictions and obvious inconsistencies in the personality organization were found. Although the patients tended to depict themselves as depleted, inadequate individuals, a need for personal assertiveness was often prominent. Marked social withdrawal was observed in the productions of the same patients who showed exaggerated attempts to contact the environment.

The ulcer patients projected an image of themselves as inflated, personally adequate, oversecure individuals. However, this group also frequently attempted to hide their defects behind social facades.

The productions of the asthmatic patients tended to be less homogeneous but the level of development was felt to be the least mature of the three.

Personality and Location of Lesion

Assuming that the experimenter has found several psychological qualities occurring with statistically greater frequency in his experimental group as compared with his control, he is still faced with the problem of whether these are the *result* of the disease process or whether, possessing these qualities, the patient is thereby more liable to the disease. Methodologically, this appears a question difficult to settle for one seems entitled to argue both ways. One can say, for instance, that regardless of the personality type of the individual in his pre-morbid state, there is a certain uniformity of conditions, similarity of experiences, repeated many times, which will result by virtue of the characteristics of the disease. Consequently, regardless of

their initial deviations, persons facing identical psychological and environmental situations will tend to develop common characteristic ways of dealing with them. However, it is possible to argue, at least theoretically, that certain personality characteristics may make one more vulnerable to certain types of physical disorders than others.

The study of Tarlau and Smalheiser is interesting because of their willingness to take a stand on this issue in an investigation in which they contrasted the personality structure of breast cancer with cancer of the cervix. They feel that "the personality patterns are *not* the results of the disease, but may have some significance in the genesis or location of the pathologic process."

What has led them to this conclusion? A pilot study conducted at the New York City Cancer Institute on Welfare Island contained a sample of 22 married women, 11 breast, 11 cervix patients. The two groups were considered equal as far as socioeconomic background and general educational level was concerned. The investigations used were a two-hour interview, the Rorschach Inkblot Test and the drawings of the human figure.

Basing their generalized conclusions on specific quantitative material from the tests, it appeared to these authors that the group with breast cancer were much less willing to accept their own inner drives and that there were greater repressive forces operating in this group as compared with the cervix group. They found that while both groups essentially rejected the feminine role and showed strong masculine feelings, that there was a more acute sexual conflict among the cervix cases. On the other hand, they felt that the cervix group were able to accept themselves to a much greater extent, showed a stronger instinctual life and a greater capacity to react to emotional stimulation. The breast group was found to be much more rigid, the cervix group more anxious.

Describing and contrasting the two groups, the authors write, "The breast patients appear to be functioning on a very primitive level as compared with the cervix patients. Severe repressive mechanisms have been used to inhibit their emotional reactivity and contact with their inner life. Little overt anxiety remains. Generally, their sexuality has been deeply repressed. In those isolated patients in which there are indications for more overt sexual

disturbance, it appears rather as a confusion regarding their sexual role and homosexual feelings.

"The cervix patients, however, present a picture that differs from the breast group primarily in the degree to which these symptoms are revealed. Consistently through the cervix patients is found a much greater degree of contact with themselves and a much greater degree of emotionality. The sexual conflicts here are much closer to consciousness. As these conflicts exert more pressure cervix patients have developed a much stronger system of paranoid traits. In this group are found violent hostility, pathological suspiciousness, irrational ideas of reference and persecution approaching hallucinatory phenomena. The cervix group might be said to be at a more genital level of psychosexual development, while the breast group has fixated at the oral level.

"Findings from the figure drawings again showed contrast between the groups. The breast patients regularly produced drawings of a very primitive, infantile level, similar to those of a child around five or six years of age. The sexual characteristics of the figures were completely lacking. Legs and arms were often omitted and the drawings appeared to be those of persons seriously cut off from reality.

"The cervix group, on the other hand, produced drawings of a much better quality. The drawings looked more like those of adults than those of children as was characteristic of the breast group. Clothing was always included in the cervix drawings, omitted in the breast.

"A comparison of the drawings by the two groups substantiates the conclusion that the breast group is functioning at the more primitive and that the cervix group is at a more advanced level. Both groups show evidence of homosexual conflict although it is more acute and closer to the surface in the cervix patients."

The authors claim that these personality patterns revealed by the Rorschach data are probably *not* the result of the cancer process itself, basing this on their findings of the existence of these problems previous to the illness as revealed by the *interview* material. They go even further in stating that there is some evidence which suggests that the personality structure may play a role in the pathogenesis of cancer of primary or secondary sex organs in predisposed individuals.

This study which shows the interrelation of the psychiatric and psychological method of examination—the interview on the one hand, the psychological tests on the other—shows an appropriate interplay of the two disciplines. From the interview independently, the authors concluded that the breast cancers appeared in women who are functioning psychologically at a primitive oral level while cervix cancers appeared in patients

who have genital fixations. The psychiatric findings or the psychological findings alone, would be of interest, but they become of much greater significance when, from independent lines of investigation, they point to similar conclusions.

Clinical Psychology in Medical Research

The use of psychological tests in the interdisciplinary study at the M. D. Anderson Hospital for Cancer Research in Houston, Texas, may, perhaps, best be described in the words of Cobb:

A Medical Psychological Fellowship Program

"A training-research program has been initiated at M. D. Anderson Hospital for Cancer Research, a division of The University of Texas, Houston, Texas. The purpose of which is a two-fold one. First, it purposes to prepare psychologists at a doctoral level, for participation in a team approach in a medical-psychological research setting. Second, it aims to contribute to fundamental knowledge of the cancer patient as a person as well as to a more comprehensive understanding of physiochemical-psychological inter-action in the disease.

"*The fellowship* consists of three phases of study—medical training, service to patients, and supervised research. The medical training course runs throughout the year and consists of three divisions, a medical orientation to cancer taught by the medical staff, a course with laboratory sessions devoted to anatomy of the central nervous system, a course in endocrinology including lectures and experimental sessions in the animal laboratory.

"*The service to patients* is done upon referral by the physicians and under the supervision of the psychiatrist. It affords the psychologist experience with patients under stress other than pathological mental disorders.

"*The research* experience is under the direct supervision of an interdisciplinary Advisory Council and the hospital staff. Research is required to contribute to two areas of endeavor now under way: behavior studies and psychiochemical-psychological investigations.

"The behavior studies are centered around practical problems presented by the medical staff. Why do some people seek prompt diagnosis and treatment when cancer is suspected? Why do doctors delay when there is some possibility of a neoplasm? Why do people detour to "Quacks" when they have an idea that malignancy is present? Why do a number of patients refuse treatment when carcinoma, etc., has been diagnosed?

"Variables in cancer behavior probably are social, cultural, and individual, as well as organic. Differences among patients possibly may be associated with their backgrounds of social experiences. An important consideration is the psychological impact of the disease itself, as well as the perceptions and valuations surrounding it, upon the cancer patient.

"Research problems which have to do with the interplay between possible endocrine imbalances in cancer and shifts in psychological responses are under investigation. The question to be investigated might be stated in three ways. Do endocrine imbalances present in cancer create physiological disorders and consequent psychological stress? Do the physiological disorders produce endocrine imbalances which in turn build psychological stress? Or, does the psychological stress initiate endocrine imbalance resulting in physiological disorders, or vice versa?

"To explore the several possibilities, a medical-biochemical-psychological approach with an inter-disciplinary research team has been initiated. Continuous biochemical assays to measure steroid hormone metabolism and other shifts in body chemicals parallel clinical records of the medical staff and repeated psychological assessments of the patients."

The significance of this particular study lies not only in the interdisciplinary aspects, but perhaps more fundamentally in the realization of these physicians and psychologists that clinical tests will never contribute as much as they might until psychologists are better oriented in the field of medicine. What was a pious hope a few years ago now becomes a reality in the courses which are planned to be given at the Anderson Hospital and while it may well be that other and different orientation courses will be developed in other places, their feasibility and the enthusiasm which is reflected both amongst the teachers and pupils is an important step in the right direction.

Conclusions

Several general impressions arise from surveying the literature in regard to personality characteristics and specific somatic disease. With the possible exception of a group of patients with cerebral lesions the personality picture is *never a uniform one for all members of an experimental group.* The most any investigator can say is that certain characteristics, measurable by his test instruments, appear more frequently in his experimental group than in his controls. Moreover, there is a similarity between those traits which are isolated as "characteristic" in *several* disease groups.

Thus, a prediction from psychological test findings that such and such a patient has such and such a disease is at best tentative . . . again with the exception of cerebral involvement.

On the other hand, the assessment or evaluation of unique personality difficulties on the one hand, and unique strengths and assets on the other for any individual patient, is exactly as valid and relevant when the patient happens to have some somatic disease as when his disturbance is manifest in some emotional problem. If psychological findings can be communicated to the physician in a nontechnical and relevant way, there must result a better understanding of the patient as a whole person.

As in any new procedure, the estimate which is put on its value is likely to represent unduly wide swings of the pendulum. Psychological tests may be too readily debunked, or conversely surrounded with an aura of infallibility. Like any other procedure, psychological tests are subject to error, and to misinterpretations. Moreover, while they are no more or less "subjective" than the reading of an X-ray plate, there is as much difference between the specialist's opinion and that of the psychological intern, when assessing test findings, as there is between the radiologist's opinion and the fourth-year medical student. Experience counts.

What do we need in order that the contributions of clinical psychological tests to the understanding of somatic disease may go further and penetrate more deeply?

First, it would seem that along the lines of the Anderson program psychologists who are to interact with physicians must have a training which is relevant to that interdisciplinary role. Whether this training should be part of the regular education of every clinical psychologist or a specialized post-graduate branch would probably depend on the amount of interest that develops in general in this overlapping field of psychological medicine.

The counterpart of this, an awareness of medical students early in their career of psychological techniques, would necessitate alterations in the current medical curriculum to include not only didactic lectures in psychology but the opportunity of the medical student to work on diagnostic problems with his psychological colleague.

A third move would be the utilizing of psychologists both in private practice and in departments of medicine by physicians

for personality diagnoses of patients who are chronically ill and for those with acute manifestations of disease. Only by such on-the-spot and directly relevant studies can demonstrate convincingly that the psychologist has much to offer in the field of general medicine. It is important that the idea of the psychologist being inevitably part of a neuropsychiatric team whose services may be utilized by the psychiatrist with reference to the severe deviations of behavior only, be dispensed with. In the words of Paul Holbrook, "Every chronically ill patient has a psychological problem." And to this one might add, "Every acutely ill patient has a psychological problem." For no dramatic change in life-situation can fail to leave some manifestation of psychological tension which, being better understood, will be better handled both by the patient himself and his physicians.

The title of this paper included the words, "fuller understanding of somatic disease." Understanding is a big word. The patient with his somatic disease requires understanding at various levels—the understanding of those who administer to his immediate problems, the tender, loving care of the nursing manuals. He needs the understanding of every physician of the fact that anxiety may undermine his physical recovery, and he needs the understanding which can be derived from such techniques as those we have mentioned whereby specific problems relating to the type of disease from which he is suffering and the problems relating to his personality makeup may be taken into account and insofar as possible successfully handled.

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A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF GLUCOSE*

STEWART C. HARVEY† AND VELMA HIGBY

Most methods for the quantitative determination of glucose and other monosaccharides involve reduction of a titrimetric reagent or potential chromogen. According to the manner of preparation of the biological sample, certain reducing contaminants may be removed to insure the specificity of the method. Occasionally, however, it is desirable to employ an analytical method that depends not upon the reducing properties of the sugar but rather upon some other property. During the development of a microcolorimetric method for glycerol (Harvey and Higby, 1951), it was discovered that the condensation of glucose with catechol in sulfuric acid yielded a highly chromogenic substance in direct proportion to the amount of glucose present. Although the method offers no advantages over the usual methods of analysis under ordinary circumstances, it may be occasionally employed when contaminating reducing substances are difficult to remove from a biological sample.

Methods. Preparation of the Biological Sample. Any method of deproteinization that does not remove glucose may be employed. Tungstic acid, trichloracetic acid or baryte-zinc sulfate precipitations are acceptable but copper-lime removes glucose. Perchloric acid does not remove glucose but interferes with the formation of the chromogen, and furthermore it may be dangerous under the conditions of color formation. If the filtrate is to be stored, it should be neutralized after the protein is removed so that glycerides will not hydrolyze in storage. Otherwise, the filtrate is adjusted to a pH of 3 and extracted three or more times with ethyl acetate to remove certain interfering organic acids and glycerides. Residual ethyl acetate may be volatilized or, better, removed with diethylether. When glycerol is present, it should be determined according to the method of Harvey and Higby (1951) and the glucose content corrected for glycerol, or glycerol may be removed by repeated evaporation with ethanol, the glycerol being entrained in the ethanol vapors. Glycogen may be precipitated and removed by adding hot ethanol until the concentration thereof is 75 per cent by

* From The University of Texas School of Dentistry, Houston. Received for publication May 20, 1953.

† Present address: Department of Pharmacology, University of Utah College of Medicine, Salt Lake City.

volume, and then refrigerating for several hours. The alcohol should then be removed by evaporation until nearly dry or by extraction with ether.

Development of the color. Prior to analysis, test tubes are selected for uniformity of diameter and wall thickness in order that heat transfer be uniform. All glassware should be scrupulously clean. Reagent and sample solutions are precooled to 0.5° C., and the tubes are immersed in an ice-water bath during the course of addition of reagents to avoid excessive premature heating upon addition of the sulfuric acid mixture. Meanwhile a concentrated sulfuric acid or oil bath under a hood is brought to a temperature slightly above 145° C., so that the temperature of the bath does not drop below 140° C. when the cold prepared tubes are immersed therein. It is preferable to prevent an excessive temperature drop by increasing the size of the bath rather than by raising the initial temperature above 145° C. The high heat capacity and water miscibility of sulfuric acid make it the bath medium of choice.

To each tube in the ice bath 1.0 ml. of the glucose-containing solution is added, after which 1.0 ml. of *freshly prepared* 10 per cent catechol solution[1] is added. Four ml. of 84 per cent sulfuric acid solution[2] are then added carefully, and the contents are gently stirred with a small glass rod. Standards are prepared simultaneously. If all solutions have been precooled, no color should appear at this time. Oxidized catechol solutions give the mixture a brown color. After the gently agitated mixtures have stood in the ice bath for a few minutes to allow them to reach the same temperature, all the tubes are simultaneously placed in the sulfuric acid bath and incubated for 10 minutes at 140–5° C. A red color develops during heating. At the conclusion of the heating period the tubes are simultaneously immersed in a crushed-ice bath. When the tubes are cool, the intensity of the color is read in a colorimeter with a lavender filter or in a spectrophotometer at 440 mμ, with a reagent blank for reference.

Results and Discussion

When aqueous solutions containing known amounts of glucose were employed in the above procedure, conformity with Beer's Law within the range 0–140 microgms. of glucose was observed. A standard curve so obtained is shown by the circles in figure I. The absorption spectrum of the chromogen is depicted in figure II. The peak at 440 mμ determines the wave length at which analyses are best made. If color selection is done by means of filters, either 420 or 490 mμ filters are satis-

[1] One gram of twice-sublimed catechol is dissolved in 9 ml. of chilled water *immediately before use.*

[2] Prepared from commercial reagent grade H_2SO_4 sp. gr. 1.8, by adding 3 vol. concentrated acid to 1 vol. water.

factory. In fact, even a green filter permits conformity with Beer's Law, but the method is less sensitive.

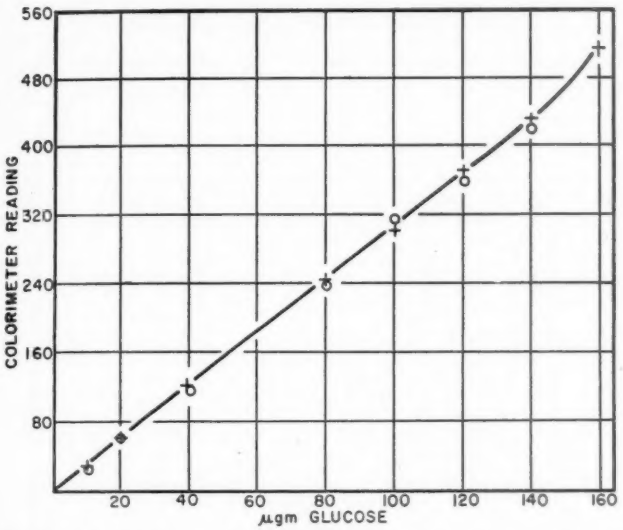

FIG. I. Line drawn through crosses is a standard curve determined from aqueous solutions of glucose. Open circles are data determined from a rat liver homogenate to which known amounts of glucose had been added. Readings were made with Klett-Summerson photoelectric colorimeter.

The circles in figure I depict results obtained from a protein-free filtrate prepared by adding known amounts of glucose to a rat liver homogenate. The homogenate was previously fermented with a small amount of yeast to ferment glucose. The activity of the yeast was destroyed by 0.04 M. iodoacetic acid, and the standard glucose was added. Unfermented glycogen was precipitated with hot 75 per cent ethanol and removed by centrifugation. Tungstic acid precipation removed the protein not already precipitated by the hot ethanol. The ethanol was then evaporated from the filtrate and water was added until the original volume of the homogenate was restored. Organic acids were removed by ether extraction. It can be seen in figure I that recovery was good (average recovery, 99 per cent) and that the curve so obtained is also rectilinear.

The conditions of temperature and period of heating have not been investigated but are probably not critical, since considerable latitude was found allowable in the glycerol analysis (Harvey and Higby, 1951). It is probable that heating at lower temperatures or using lesser concentrations of sulfuric acid would reduce the chromogenicity of potentially interfering substances.

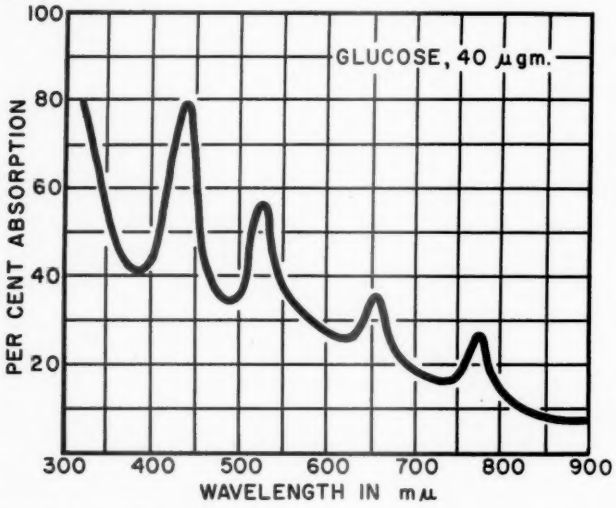

FIG. II. Absorption spectrum of glucose chromogen, determined with a Beckman model DU spectrophotometer. Double-distilled water was used as a reference, but all values were corrected for the reagent blank.

Glycerol and certain carbonyl containing substances can also give rise to a chromogen (Harvey and Higby, 1951) and thus interfere with the method unless the filtrate is prepared according to the above. The chromogenicity of such substances is generally low, with the exception of glycerol and other sugars. With the exception of sugars, interfering substances are easily removed. Furthermore, under many circumstances the amount of interfering substances compared to glucose is nearly negligible.

Summary

A micro method for the quantitative colorimetric estimation of glucose is described. Glucose and catechol are heated in sulfuric acid at 140–5° C. to give a red color. The method can be employed in the presence of reducing substances which would render the usual methods inaccurate.

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Harvey, S. C., and V. Higby: A Microcolorimetric Method for the Quantitative Determination of Glycerol. *Arch. Biochem.*, 30: 14–24, 1951.

EFFECT OF A PROTEIN-FREE BACTERIAL PYROGEN ON LEUKOCYTIC COUNTS IN IRRADIATED MICE*

DAVID T. HENKEL, ROY B. MEFFERD, JR.,† AND JOHN B. LOEFER‡

Certain prophylactic measures such as pretreatment with cysteine (Patt *et al.*, 1949), glutathione (Chapman *et al.*, 1949), cyanide (Bacq *et al.*, 1950), ACTH (Betz, 1951; Taber, 1951), desoxycorticosterone (Mirand *et al.*, 1952; Ellinger, 1947), and lowered oxygen tension (Dowdy *et al.*, 1950), were shown to be effective in reducing mortality of irradiated animals (*cf.*, Porter, 1952; Smith *et al.*, 1950; Stranbe *et al.*, 1949). The work of Jacobson *et al.*, 1951), opens the way for effective chemotherapeutic measures. They have shown that shielding of the spleen during irradiation results in a marked decrease in mortality. Use of intraperitoneal implantation of splenic tissue or of embryonic tissue suspensions, and related techniques after irradiation likewise does so. Their evidence indicates that a humoral factor is responsible for the recovery. They (1952) have also shown that survival roughly parallels hemopoietic recovery with dosages up to 1,025 roentgens (r). The development of major sites of ectopic blood formation is not essential and probably is not the decisive factor in the recovery of the hemopoietic tissue, or in the survival of spleen-shielded animals. The influence of specific stimulation of erythroid elements was earlier investigated using phenylhydrazine (Jacobson *et al.*, 1949) and low oxygen tension (Schack and MacDuffee, 1949) and in both cases stimulation prior to irradiation enhanced resistance.

The suggestion of Jacobson *et al.* (1951) that irradiated tissue may be stimulated to an earlier recovery appeared to offer an excellent experimental approach to the problem. It seemed to us that stimulation of the reticulo-endothelial system, be-

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† Fellow in Cancer Research of the Damon Runyon Memorial Fund.

‡ Present address, office of Naval Research, Pasadena, and Department of Zoology, University of California at Los Angeles.

lieved by Jacobson to be implicated in recovery from radiation injury, with Piromen[1] warranted investigation. Piromen is a purified bacterial pyrogen which stimulates a leukocytic response very similar to that produced by treatment with ACTH (Clemente, 1950 and 1951; Randolph and Rollins, 1950). The mechanism of action is unknown, but it is believed to act in part on the adrenal cortex and other endocrine glands (Windle *et al.*, 1950). There is evidence, however, that its action is not identical with that of ACTH (Lewis and Page, 1948; Stuart, 1951); Soylemezoglu and Wells, 1951). Prolonged administration of Piromen induces extramedullary hemopoiesis in the spleen and liver (Windle and Wilcox, 1950). Such treatment also stimulates the reticulo-endothelial system and the myeloid hemopoietic tissues, as well as moderately stimulating lymphoid tissues (Windle *et al.*, 1950).

Materials and Methods. Various agents were evaluated in preliminary studies for hemopoietic stimulatory properties, including killed bacterial suspensions, non-homologous leukemic cells, and pyrogenic compounds such as tetrahydro-beta-naphthylamine (Cramer, 1920), Reticulose (Thompson, 1952), and Piromen. The latter was the compound of choice. It was superior to the others both in the degree of response, *i.e.*, stimulation of splenic tissues and extent of leukocytosis, and in its rapidity of action. Furthermore, when administered in small doses, its stimulatory effect is separated from its pyrogenic effect (Randolph and Rollins, 1950; Kirkendall *et al.*, 1951). In the marked leukocytosis which Piromen induces, following administration of small doses orally, the ratio of granulocytes to agranulocytes remains constant in rats (Clemente, 1950).

The HN strain of Rockefeller Institute Swiss albino mice used in these experiments was maintained in an air-conditioned animal room at 78° F. The animals had free access to Purina Laboratory Chow and water. Twenty-one 12-week-old males were segregated at random into three equal groups. Groups 2 and 3 received Piromen in their drinking water before and after irradiation, respectively. Group 1 (control) received no Piromen. Total consumption of Piromen per mouse was 1.92 gamma (over a 5-day period) and 9.05 gamma (over a 16-day period) for Groups 2 and 3, respectively. This constituted an average daily dose per mouse of 0.384 and 0.565 gamma in each case. Administration of Piromen in the drinking water was convenient, but it was impossible to estimate the actual dosage absorbed. Undoubtedly, a considerable part of that imbibed was eliminated or destroyed by the intestinal flora, and the actual effective dose was thus very small.

[1] Furnished through the courtesy of Dr. W. F. Windle, Baxter Laboratories, Morton Grove, Illinois; trade name for a sterile non-protein, non-anaphylactogenic bacterial pyrogen.

A Picker 30 ma, United States Army Field X-ray unit was used. Irradiation factors were: 86 kvp., 4 ma., 2.5 mm. Al and 0.5 mm. Al equivalent (inherent in machine) filters, 32.5 cm. target-center of field distance, 0.125 mm. Cu. half value layer, and 10 r/minute dose rate. Exact location of the 25.4 cm. diameter field was determined before irradiation by means of a fluorescent screen. The most homogeneous portion of the field was utilized by placing the animal-radiation chamber under the cathode side of the tube. The anode-heel effect was, therefore, negligible. Measurements made with a 100 r Victoreen Condenser r-meter in all parts of the field during irradiation revealed that the dose delivered in air and inside the radiation box did not vary by more than 5 per cent in any part of the field. To avoid possible stress and side effects due to hormonal disturbance, the animals were not confined to any one particular area during irradiation, but moved freely in the chamber. With small animals such as mice, body irradiation may be considered to be uniform throughout. The irradiation chamber was constructed of 1/8 inch lucite with ample ventilation ports. The total irradiation dose of 350 r was determined by summing r-readings, corrected for room temperature and barometric pressure, at 7-minute intervals.

Blood counts have been reported to be a reliable method for gauging the damage resulting from various radiation dosages (Price, 1951), although the work of Furth *et al.* (1951) indicates that high doses (600–800 r) alter the volume relationships of the plasma, so that direct counting techniques following such doses do not give a true picture of the total numbers of cells present. Doses between 300 and 400 r, however, do not change the volume relationship (Supplee *et al.*, 1951). The selection of a suitable radiation dose for these experiments entailed several considerations including a dose which would not alter plasma relationships appreciably. Another factor considered was the differential sensitivity to radiation of the various leukocytic types and of the hemopoietic tissues. Radiation damage to hemopoietic tissue becomes manifest and is reflected in the peripheral blood leukocytes shortly after exposure. Radiation damage to the circulating elements, however, is even more rapidly evidenced. Thus peripheral lymphocytes show damage within fifteen minutes (Lawrence *et al.*, 1948). Since the average duration of lymphocytes in the blood is twelve hours (Reinhardt, 1946), there is a short period during which primary damage is detectable, but thereafter the picture is more complex as replacement mechanisms fail. Neutrophils are more resistant and changes in their number do not parallel that of the lymphocytes. Free hematopoietic cells such as these, however, are not necessary for the production of the radiation-recovery factor (Jacobson, 1952). To avoid threshold sensitivity differentials, as well as for the other reasons given above, a total body dose of 350 r was administered.

A small segment of the tail was dipped in alcohol, snipped with sterile scissors, and a drop of blood taken directly into the blood pipette. To avoid anemia and possible infections, the bleeding was stopped by dipping the tail in equal parts of powdered aluminum sulfate and Combiotic.[2] All animals

[2] Trade name of mixture of dihydrostreptomycin, crystalline penicillin G procaine, and buffered penicillin G sodium. Produced by Chas. Pfizer & Co., New York.

were treated in this manner so that any effect such treatment may have had upon the blood picture was equalized. Standard blood-counting procedures were employed for making the leukocytic counts (Kracke, 1947). Counts were recorded as shown in table 1. This tail-snip procedure resulted in a slight initial rise in the total leukocytic count. For this reason, experiments were not commenced until the tails of all mice had been snipped daily for a period of several days. No detectable anemia resulted.

Results and Discussion

Administration in the drinking water at the rate of about 0.5 gamma Piromen/day/mouse effected, within a few days, a leukocytosis, which was reasonably uniform for all classes of leukocytes, and in which there was a three- to four-fold increase in number of circulating cells. Splenic tissue was observed to be myeloid and in a somewhat hyperplastic condition. In preliminary experiments the degree of leukocytosis induced was found to vary, due probably to the actual dose imbibed, sometimes resulting in counts as high as 48,000 leukocytes/mm_3. These leukocytic numbers, once attained, remained relatively constant and were not subject to sharp daily fluctuations. Administration of approximately 0.5 gamma/day/mouse to another group of mice for a thirty-day period resulted in no gross deleterious effects, except that the spleen, liver and marrow were hyperplastic.

None of the animals in Groups 1 and 3 died as a result of the irradiation, but one in Group 2, displaying symptoms typical of radiation sickness, died on the fourteenth day.

It appears that the reticulo-endothelial tissue of the spleen is involved in some way in the production of the radiation-recovery factor. In experiments where total body doses of 1,025 r were delivered to mice, and an increment of this was allowed to fall upon the unshielded spleen, it has been demonstrated that dosages sufficient to destroy the lymphoid tissue, but not the more radioresistant reticulo-endothelial tissue of the spleen, hastened hemopoietic recovery (Jocobson *et al.*, 1951). Further evidence of these workers indicated that there is a relationship between the dose of splenic tissue administered and both survival and hemopoietic recovery. It is logical to assume that a stimulation of the splenic tissue, as evidenced

by leukocytic counts, might result in an increased production of the factor, either directly or indirectly. Yet it is seen from table 1 that irrespective of the leukocytic count at the time of irradiation, the resulting blood picture is identical afterwards and recovery proceeds at the same rate in both cases (Groups 1 and 2). Post-irradiation treatment with Piromen failed to alter the blood picture until the thirteenth day after irradiation, at which time there was a steady trend toward a more rapid hemopoietic recovery. By the twenty-third day the leukocytic count was normal, while counts in the other two groups were still much below normal. These results must be accepted with caution, since extended tissue studies were not made, and the hematological picture may not actually reflect accurately the true hemopoietic status.

TABLE 1

Effect of Piromen Treatment on Average Leukocytic Counts of Irradiated Mice

	Group 1 No Piromen Treatment Control			Group 2 Piromen Treatment Before Irradiation			Group 3 Piromen Treatment After Irradiation		
DAYS	WBC	Lympho-cytes*	Granulo-cytes*	WBC	Lympho-cytes*	Granulo-cytes*	WBC	Lympho-cytes*	Granulo-cytes*
–7	10350	83.8	13.4	9953	68.8	26.6	9654	78.0	18.6
–6	13354	78.6	18.8	15575	75.4	22.5	10400	81.2	16.4
–4	8891	82.0	16.5	7937	79.3	34.7	8952	82.0	17.0
–3	11210	80.1	17.9	11875	75.1	27.7	13887	78.0	19.1
–1	10980	71.3	16.9	28093	90.7	7.7	11220	80.1	17.6
0 X-irradiated 350 r	13073	70.1	22.1	26178	86.4	10.7	12110	71.2	22.3
1	4156	43.2	54.0	3395	39.6	57.7	3875	53.0	46.5
2	2075	59.8	39.8	2167	54.0	43.2	2571	54.3	45.2
4	1979	69.0	13.4	1854	77.6	17.4	2405	60.2	38.1
6	2240	66.8	31.6	2458	77.6	19.1	2732	77.1	22.2
8	2700	55.0	41.3	3387	54.6	43.8	1571	80.2	20.0
10	1979	69.0	14.4	1854	77.6	17.4	1450	82.4	16.0
13	4589	69.5	25.0	3708	80.6	15.4	5748	69.2	30.1
16	4733	73.1	25.9	5062	75.3	23.8	6437	66.8	32.1
19	4466	64.1	33.9	4270	64.1	34.8	8145	51.2	45.4
23	5409	62.6	36.1	5937	59.5	40.1	10583	76.1	23.1

* Percentages from differential counts.

Note: Standard deviations of 45 normal leukocytic counts (including those in Groups 1 and 3 above prior to irradiation): Total count: 1770; Lymphocytes: 5.9 per cent; Granulocytes: 9.9 per cent.

Several possibilities for the poor prophylactic response may be noted. The survival factor may be destroyed *per se* by 350 r regardless of the amount present at the time of irradiation. On

the other hand, it may be that it is not produced in increased quantities at all following Piromen stimulation. Possibly alteration of the dosages of either Piromen or X-radiation would yield more significant results. In another experiment of the same design Piromen was injected i.p. but the results were confusing and inconsistent. It should be noted that when the therapeutic substance is injected into the animal, interpretation of results is difficult in view of Ellinger's (1952) report that physiological saline alone has protective value.

Summary

Oral administration of Piromen, a protein-free bacterial pyrogen, markedly stimulated the hemopoietic tissues and spleens of mice. The normal percentages of the various kinds of leukocytes were relatively unchanged. To determine the influence of such stimulation upon the hematological picture in X-irradiated mice, Piromen was administered to one group before irradiation, to another afterward, while a third group received none.

Although there was no apparent prophylactic value of Piromen, therapeutic oral doses resulted in an earlier recovery of the hemopoietic tissues as evidenced by numbers of circulating leukocytes. Starting on the thirteenth day after irradiation those animals receiving therapeutic treatment began to exhibit a rise of leukocytic counts, and by the twenty-third post-irradiation day the count was normal, while the counts in the other two groups were still very low. Undoubtedly, the hemopoietic tissues play an important part in recovery from radiation injury, and any speed-up in the processes of recovery would be beneficial.

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ELECTRON MICROSCOPIC STUDIES OF THE VIRUS OF GERMAN MEASLES (RUBELLA)

REGINALD L. REAGAN, NINALEE STRAND, AND ARTHUR L. BRUCKNER*

German measles (rubella) is an acute infectious disease and a distinct disease entity. Wagner (1829) first separated it from measles and scarlet fever. The disease has been transmitted to children by the inoculation of filtered nasal washings (Hiro and Tasaka, 1938). This virus may be transmitted experimentally to young Macaca rhesus monkeys with blood taken from human cases. This was accomplished by Hess (1914). Habel (1942) reported the successful transmission of the disease to Macaca mulatto rhesus monkeys. Nasal washings and defibrinated blood was used. The incubation period was from 7 to 9 days. Pyrexia was slight and some leukopenia occurred. The exanthem consisted of sparse pink macules chiefly on the trunk and there was no enanthem. He found no cross immunity between measles (morbilli) and German measles (rubella). Apart from filterability pointing to a virus, little is known about this agent. It has been grown on the chorioallantoic membrane of chicken embryos by Habel (1942). This experiment was undertaken to photograph the virus by electron microscopy such as was accomplished with the virus of measles (morbilli) by Reagan *et al.* (1952).

Materials and Methods. Defibrinated venous blood collected from a patient with German measles at the time of fever and rash was obtained from Janis Campbell (age 20 years) at her home in Washington, D. C. This patient was bled from the marginal vein, and the defibrinated blood was stored at –60° C. for 6 weeks before initiating the experiment. Since the virus may be quite susceptible to heat, it was kept chilled whenever possible, and all glassware was chilled before being used.

Monkeys (Macaca rheus) weighing from 2 to 3 pounds were used throughout the experiment. All animals were housed for the period of the experiment in individual cages, and normal monkeys were kept in a separate room from the infected ones. Isolation precautions were maintained in handling the animals. The animals were carefully examined each day for

* From the Virus Laboratory, Live Stock Sanitary Service, Maryland State Board of Agriculture, College Park, Maryland. Received for publication April 18, 1953.

developments of changes in the skin, eyes, nose, and inside of the mouth. Rectal temperatures were taken each moning, but no blood was drawn for blood leucocytic examination. The animals were caught in such a manner as to minimize exertion and excitement, and they were allowed to relax before the temperatures were taken. A diet consisting of milk, bananas, oranges, lettuce, and one vitamin pill, was provided daily throughout the experiment.

Experimental. Three monkeys (Nos. 1, 2, and 3) were lightly anesthetized with ether and were exposed with 2 ml. intranasally and 2 ml. subcutaneously and dermally of defibrinated blood from the measles case stated above. Three monkeys (Nos. 4, 5, and 6) were injected with normal defibrinated blood in the same manner. On the 6th day post inoculation the rectal temperatures of monkey No. 1 reached 104.0° F., monkey No. 2 reached 103.4° F., and monkey No. 3 reached 103.0° F., and typical symptoms of German measles were noted. No Koplik's spots were seen on the buccal mucosa of the 3 monkeys. An exanthem consisting of sparse pink macules along the chest and under the right arm covering an area of about 6 cm. was noted in monkey No. 1 whose rectal temperature reached 104.0° F. A few sparse pink macules along the trunk and chest of monkeys 2 and 3 were seen although their rectal temperatures were not high. Monkeys 1, 2, and 3 were each bled from the heart for 20 ml. of blood. The monkeys survived the bleedings, and daily observations were conducted for a period of 21 days. Monkeys 4, 5, and 6 remained normal and, on the 6th day post inoculation, were each bled from the heart for 20 ml. of blood.

The infected blood from monkeys 1, 2, and 3 and the normal blood from monkeys 4, 5, and 6 were kept at –4° C. for 2 hours. The blood specimens were then centrifuged for 5 minutes at 2,000 r.p.m. in a chilled angle centrifuge. The serum was removed from the infected blood and pooled. The serum was also removed from the normal blood and treated in like manner. Each pool was filtered through type ST size L3 Seitz filters. The filtrates were then subjected to centrifugation under refrigeration in a Spinco ultracentrifuge for 2 hours at 44,770 r.p.m. The temperature of the refrigerated outer jacket stayed constant at –10° C. during the centrifugation. After the 2 hour centrifugation period, the supernatants from the specimens were discarded, and the sediment from each specimen was resuspended in 1.0 ml. of chilled physiological saline. Several drops of each suspension were placed on several parlodion film supports which had been prepared 72 hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium (Williams and Wyckoff, 1946) at arc tangent 2/10 and examined under the R.C.A. electron microscope, type E.M.U.

The balance of the material of the infected serum was injected intranasally, intradermally, and subcutaneously into 2 susceptible monkeys (Nos. 4 and 5) and into 2 monkeys (Nos. 7 and 8) which had been passively immunized against measles (morbilli) by intramuscular injections with 2 ml. of immune serum globulin, Pitman-Moore (Lot No. 170A081). Monkey No. 6 (control) showed no symptoms of German measles throughout

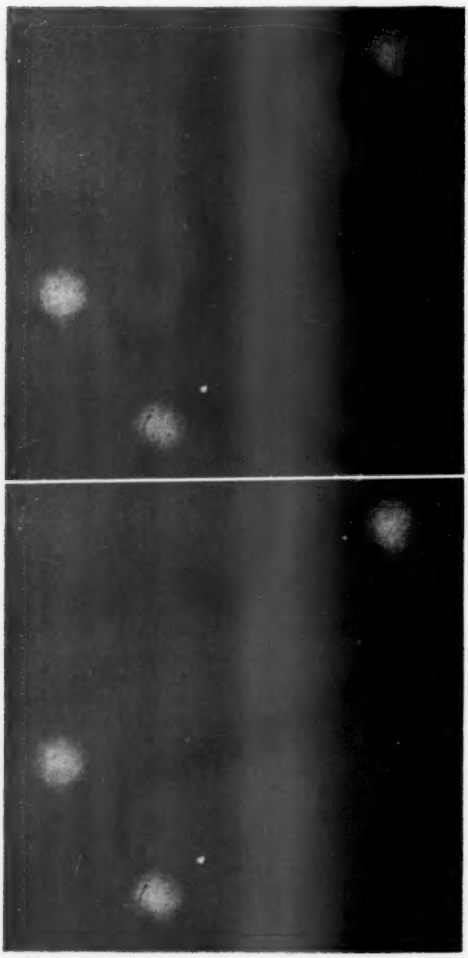

FIG. I. German Measles Virus (Rubella) shadowed with chromium at arc tangent 2/10, X 43,000.

the experiment and was discarded after a 21-day observation period. On the 5th day monkey No. 4 shwoed an exanthem consisting of sparse pink macules along the chest and under the right arm. The monkey's temperature reached 103.6° F. and no Koplik's spots were seen. Monkey No. 5 appeared normal and showed no rash or high temperature up to a 21 day observation period.

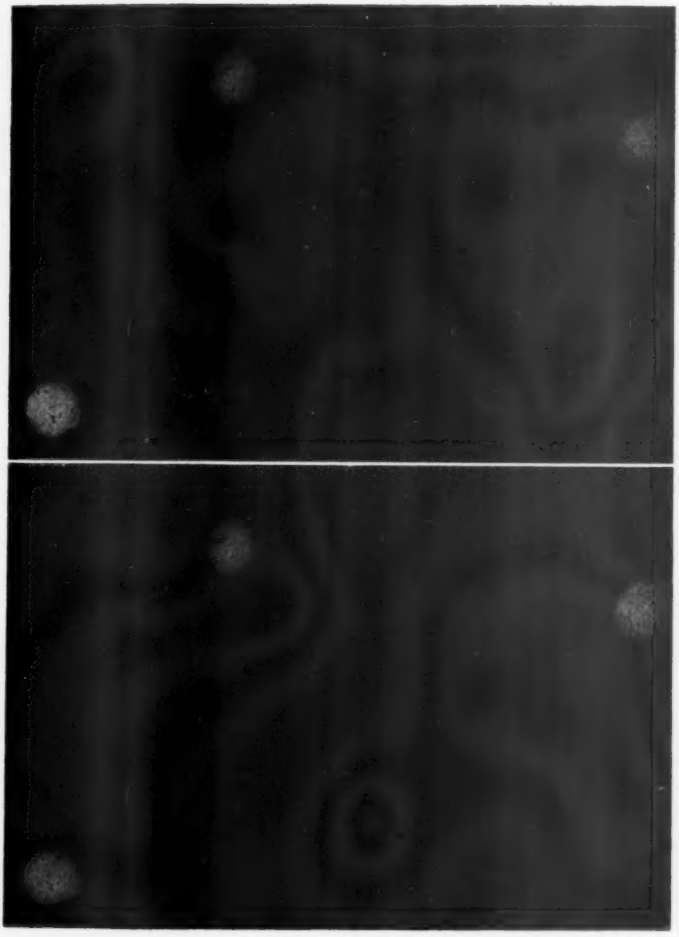

FIG. II. German Measles Virus (Rubella) shadowed with chromium at arc tangent 2/10, X 23,500.

Monkey No. 7 immunized previously with measles globulin (morbilli) showed an exanthem consisting of sparse pink macules along the trunk and no Koplik's spots could be seen on the buccal mucosa. Monkey No. 8 appeared normal and showed no rash or high temperature up to a 21 day observation period.

Upon electron microscopic examination of the concentrated suspensions from the infected serum, uniform virus like particles were observed. They were present in practically all of the 3,000 microscope fields examined.

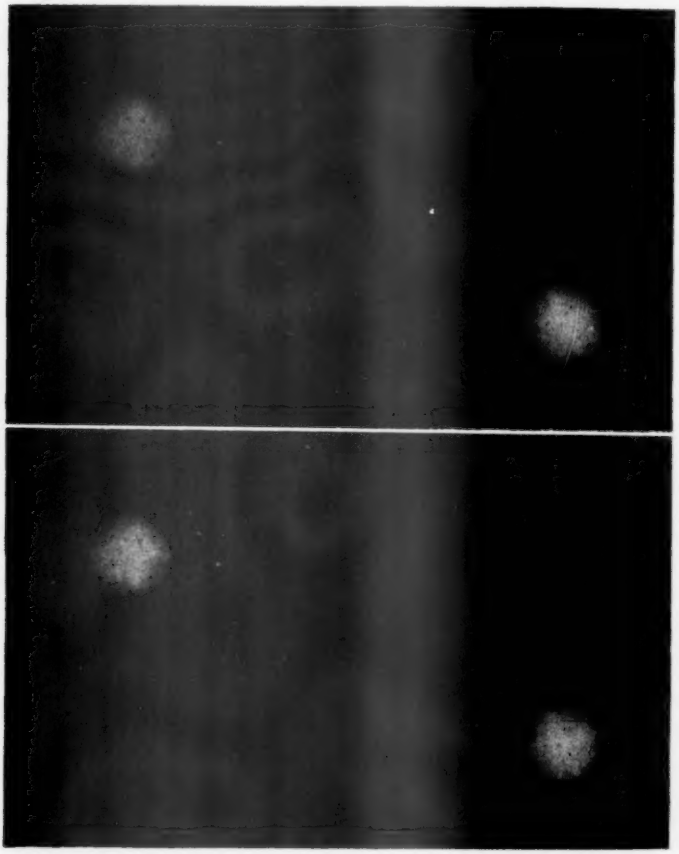

FIG. III. German Measles Virus (Rubella) shadowed with chromium at arc tangent 2/10, X 71,000.

Figures I, II, and III are electron micrographs showing these virus-like particles. The majority of these particles are round in shape and measure 150 to 160 mμ by direct measurement. No virus-like particles were seen in the concentrated normal serum in approximately 3,000 fields examined by electron microscopy.

Summary

Studies by electron microscopy of serum of monkeys infected with the virus of German measles show the virus to be round, with an occasional particle having an irregular contour. The virus particles have a diameter of 150 to 160 mμ. These bodies could not be demonstrated in normal monkey serum subjected to the same procedure of concentration and electron microscopic examination. The virus in the concentrated material was infectious for monkey No. 4 and also infectious for monkey No. 7, which was passibely immunized against measles (morbilli). No cross immunity between measles and rubella has been found by other research workers and in a previous paper Reagan and coworkers (1952) estimated the measles virus (morbilli) to have a diameter between 90 to 100 mμ. In morphology the German measles virus (rubella) seems to be much larger in diameter than the virus of measles (morbilli).

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COMPARISON BY ELECTRON MICROSCOPY OF ANOPHELES "A" AND ANOPHELES "B" VIRUSES

REGINALD L. REAGAN, NINALEE STRAND, AND A. L. BRUECKNER*

During the course of studies on the epidemiology of jungle yellow fever in Eastern Colombia, Dr. Manuel Rocca-Garcia and his associates caught a large number of wild mosquitoes and inoculated white mice with the object of attempting to recover virus. The Anopheles "A" virus was isolated from a group of 59 *Anopheles (Kerteszia) boliviensis* which had been captured on August 14, 1940 in El Horizonte, Villavicencio. The mosquitoes were ground in serum-saline and 12 adult white mice were inoculated intracerebrally. Eleven of the inoculated mice brains were removed using sterile techniques, ground with serum-saline, and passed to new groups of mice (Bugher *et al.*, 1944). The Anopheles "B" virus was isolated from a group of 21 female *Anopheles (Kerteszia) boliviensis* captured on November 29, 1940 during a study at La Cuchilla. These mosquitoes were ground in 2 ml. of serum-saline and supernatant was injected intracerebrally into 12 adult white mice. Only 2 of these became ill; one died, and the other was used for brain passage to a new group of adult mice (Bugher *et al.*, 1944; Roca-Garcia *et al.*, 1944). These two viruses were serially passed later by research workers at the Rockefeller Foundation in New York.

The viruses were furnished by the American Type Culture Collection in Washington, D.C. The Anopheles "A" virus stain had been passed 165 times intracerebrally in white mice and the Anopheles "B" virus strain had been passed 175 times intracerebrally in white mice.

Experimental Materials and Methods. Ten Swiss albino mice (3-week-old) were inoculated intracerebrally with 0.03 ml. of lyophilized mouse brains (Anopheles "A" strain of the 175th mouse passage). The mice showed roughening of the coat, followed by paralysis of the hind quarters, and then paralysis of the fore quarters after an incubation period of 5 to 7 days. When symptoms appeared, the mice from each group were sacrificed

* From the Virus Laboratory, Live Stock Sanitary Service, University of Maryland, College Park, Maryland. Received for publication May 23, 1953.

separately, and the brains were removed aseptically. Ten mice were injected intracerebrally with 10 per cent suspensions of normal mouse brains. No symptoms were noted in these normal controls. These 10 mice were sacrificed on the sixth day post inoculation. The brains were removed aseptically as above. Pools of Anopheles "A" and Anopheles "B" infected mouse brains and of normal mouse brains were ground with alundum and diluted to 10 per cent suspensions with physiological saline. The 3 suspensions were then centrifuged for 5 minutes in an angle centrifuge at 2,000 r.p.m. The supernatants were removed from all 3 specimens and filtered through type ST size L3 Seitz filters. The filtrates were then subjected to centrifugation using a Spinco ultracentrifuge under refrigeration for 2 hours at 44,770 r.p.m. The temperature of the refrigerated outer jacket stayed constant at –10° during centrifugation. After the 2-hour centrifugation period the supernatants from all 3 specimens were discarded, and the sediment from each specimen was resuspended in 0.6 ml. of distilled water. Several drops of each suspension were placed on parlodian film supports which had been prepared 26 hours previously. After all excess fluid had been removed with small capillary pipettes, the films were dried and shadowed with chromium (Williams and Wyckoff, 1946) at arc tangent 2/10 and examined under the R.C.A. electron microscope, type E.M.U. The balance of the material of each suspension was injected intracerebrally into each of six 3-week-old Swiss albino mice.

Results

The mice injected with the concentrated infected material of the Anopheles "A" strain showed nervous symptoms after a period of 6 days. The mice injected with the concentrated material of the Anopheles "B" strain showed nervous symptoms after a period of 7 days. Mice from each group were sacrificed and the brains were removed aseptically, pooled, ground, and diluted to a 10 per cent suspension. The suspension from the Anopheles "A" mice was injected intracerebrally into 4 unvaccinated mice and into 4 mice which previously had been immunized intramuscularly with Anopheles "A" virus. After 7 days the unvaccinated mice showed nervous symptoms; while the immunized mice appeared normal. This confirmed the concentrated virus to be Anopheles "A" virus. The suspensions from the Anopheles "B" infected mice was also injected intracerebrally into 4 unvaccinated mice and into 4 mice which previously had been immunized intramuscularly with Anopheles "B" virus. After 8 days the unvaccinated mice showed nervous symptoms; while the immunized mice appeared normal. This

confirmed the concentrated virus to be Anopheles "B" virus. The mice injected intracerebrally with the concentrated suspension of normal mouse brains appeared normal and were discarded after a 14-day observation period.

Upon electron microscopic examination of the concentrated suspensions of the brains of the normal mice, no virus-like particles could be seen. These controls were screened very carefully. Upon examination of the concentrated suspensions from the infected brains of the Anopheles "A" virus, uniform virus-like particles were demonstrated as shown in figure I. These

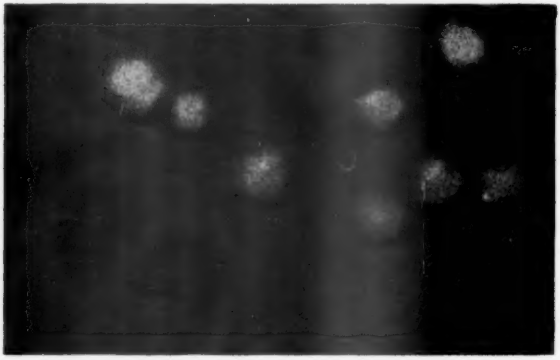

FIG. I. Electron micrograph of the Anopheles "A" virus. Shadowed with chromium at arc tangent 2/10, X 64,000.

bodies measure 110 mμ by direct measurement and are cube shaped with an irregular contour. Upon examination of the concentrated suspensions from the infected brains of the Anopheles "B" virus uniform virus-like particles were demonstrated as shown in figure II. These bodies measure 110 mμ by direct measurement and are similar in size and structure to the Anopheles "A" virus. Both viruses resemble the West Nile virus described by Reagan *et al.*, 1951.

Summary

Studies by electron microscopy of the brains of mice infected with Anopheles "A" virus show the virus to be cube shaped

FIG. II. Electron micrograph of the Anopheles "B" virus. Shadowed with chromium at arc tangent 2/10, X 64,000.

with a diameter of 110 mμ in comparison to the Anopheles "B" virus which is similar in size and shape. Both virus bodies resemble the West Nile virus bodies. These bodies could not be demonstrated in concentrated normal mice brains subjected to identical procedures of concentration and examination. The virus in the concentrated infectious material from Anopheles "A" was confirmed to be Anopheles "A" virus; the concentrated infectious material from Anopheles "B" was confirmed to be Anopheles "B" virus.

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A RADIO-TRANSPARENT POST-MORTEM TABLE

R. H. Rigdon and A. P. Freund*

The need for a more efficient method for the correlation of radiographic changes with pathologic lesions has been solved in our institution by the addition of an aluminum top to a conventional motor-operated X-ray table as shown in figure A. This table is a G. E. Maxicon presented to the Medical Branch

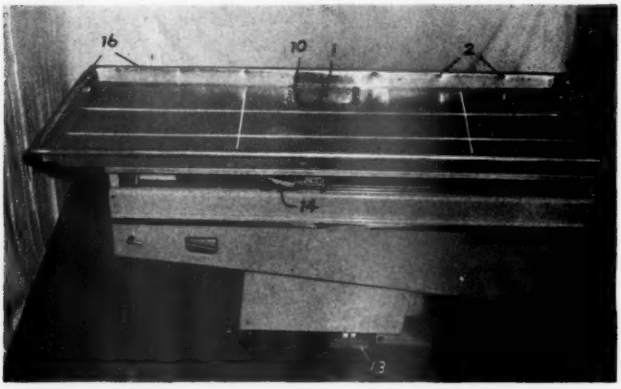

Fig. A. The combination X-ray-necropsy table in horizontal position. (1) Sink, (2) Two of the 16 water spray nozzles, (3) Water outlet, (10) Water inlet nozzle, (13) Pedals, one for elevation and one for lowering table, (14) Crank for bucky, (16) ¾-inch brass water line, (18) Scribed markings to indicate extreme positions of bucky.

for a developmental project by the X-ray Department of General Electric through the courtesy of Mr. George Dettman, our local representative.

The top is made from 1/16 pure aluminum and is built around a ¾-inch brass water line (Fig. A, 16). The dimensions of the aluminum pan are 90 x 40 x 3 inches. There are 16

* From the Department of Pathology and the Technical Apparatus Shop, The University of Texas Medical Branch, Galveston. Received for publication May 24, 1953. We acknowledge the assistance given to us by the Department of Radiology in the planning of this table. Especially do we thank Doctors Rosen, Rude, and Caroline Rowe. Mr. George Dettman of the G. E. X-ray Department also contributed greatly to the success of this project and we express our appreciation to him.

water spray nozzles ⅛-inch pipe size, 45 degrees Zirck grease fittings with the ball valve removed (Fig. A, 2) which permits water to cover the top of the pan at all times. A sink 12 x 8 x 8 inches (Fig. A, 1) serves as a receptacle for water and sponges. The pan is mounted so that the drainage is toward the outlet (Fig. A, 3) when the table is in the horizontal position. This

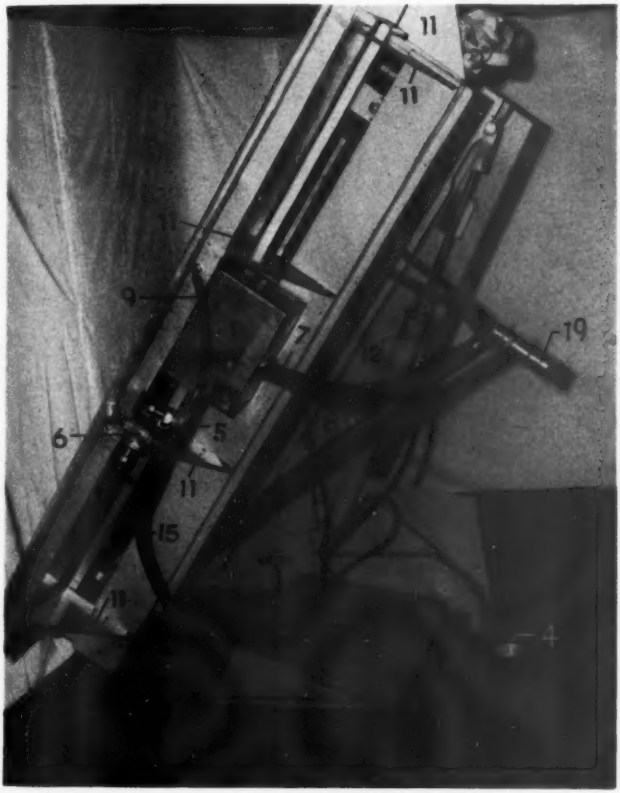

FIG. B. The combination X-ray-necropsy table in a partly raised position to show water and drainage systems and the motor-driven hydraulic system. (1) Sink, (4) Floor drain, (5) Supply valve for sink, (6) Supply valve for spray nozzles, (7) Drain for sink, (8) Drain from table pan, (9) Flexible water hose between valve and sink, (11) Five supporting brackets, (12) Hydraulic cylinder, (15) Flexible water hose connected to the water line in the floor, (17) Motor for hydraulic system, (19) Drain outlet.

drain and its connections (Fig. B, 8 and Fig. A, 3) are so constructed that when the table is moved from the vertical to the horizontal position it automatically lines up with the floor drain (Fig. B, 4).

The top of the X-ray table was removed and the aluminum pan attached by the same four bolts that held the original table top plus five additional brackets (Fig. B, 11 and Fig. C, 11).

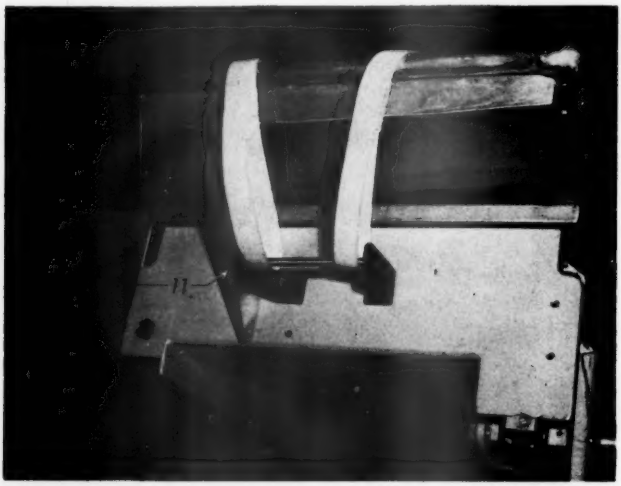

FIG. C. X-ray-necropsy table to show (11) brackets and shoulder straps.

The pan extends over one side of the X-ray table for 12 inches and over the head of the table for 12 inches (Fig. D).

The water supply is arranged so it can be turned on for the 16 spray nozzles (Fig. A, 2) and/or the supply nozzle for the sink (Fig. A, 1). The valve (Fig. B, 5) is for the sink and the valve (Fig. B, 6) regulates the water for the spray nozzles. A flexible tube (Fig. B, 9) connects with the valve (Fig. B, 5) and the inlet nozzle (Fig. A, 10) to supply water for the sink (Fig. A, 1). The water line (Fig. B, 15) is connected by a flexible hose to the water connection located in the floor beneath the table.

The drain (Fig. B, 7) from the sink (Fig. A, 1) is connected to the drain (Fig. B, 8) from the water outlet (Fig. A, 3) which enters the connection (Fig. B, 19) and then empties into the floor drain (Fig. B, 4). This drainage tube clears the floor drain when the table is elevated and automatically returns when the table is lowered.

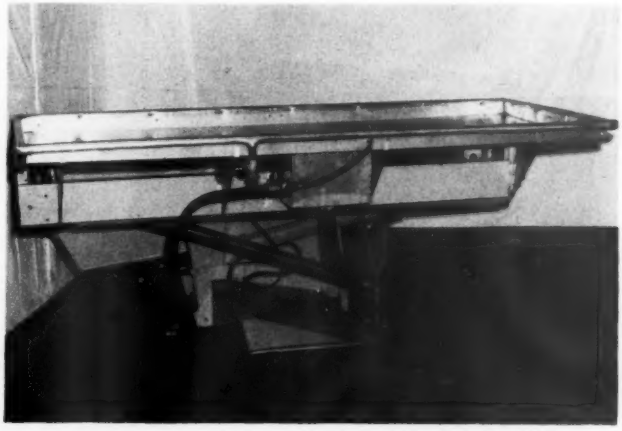

FIG. D. X-ray-necropsy table in horizontal position to show floor drain and water connections. Note the extension of the aluminum pan beyond the head and to the latter side of the table.

The table is elevated by a motor-driven (Fig. B, 17) hydraulic system (Fig. B, 12). The speed is controlled by two foot pedals (Fig. A, 13), one for elevating and the other for lowering the table. The bucky can be moved from one end of the table to the other by a crank (Fig. A, 14). A bracket is mounted to the head of the X-ray table (Fig. C) for attachment of heavy webbing that is used for the shoulder straps.

The X-ray table with the reciprocating bucky and a mobile type X-ray generator are operated from a 115-volt convenient outlet.

The preparation of the body for radiographic examination is a simple procedure. It is placed on the table and a strap is put beneath each axilla (Fig. E). The position of the body is

FIG. E. The body is suspended by shoulder straps. The position of the body is determined by scribed markings.

adjusted so the neck lies at a point corresponding to the upper margin of the bucky and the mid-line of the body corresponds to the mid-point in the bucky. These positions are readily determined by scribed markings on the aluminum table top (Fig. A).

Radiographs may be made with the body either in the horizontal or vertical position. Conventional bucky or non-bucky radiographic techniques are used employing a mobile X-ray unit (Fig. F). The bucky grid is removable for non-bucky radiographs, *i.e.*, vertical chest.

FIG. F. A radiograph is being made of the pelvis.

The necropsy may be immediately performed following the taking of the radiographs after the removal of the shoulder straps. Water and drainage is adequate on this table for the performance of the examination.

Discussion. This combination of a radiographic and necropsy table eliminates the necessity for the handling of a body twice in order to carry out these two types of examinations. The time required to process one or more radiographs is approximately fifteen minutes. An autopsy may be performed on this table as readily and with equal ease as upon any other type of autopsy table.

The advantages that are immediately available with such facilities as this combination of X-ray-necropsy table are numerous. Let us briefly consider some of these. To the pathologist this offers a rapid method for the diagnosis of tuberculosis in cases that die shortly following hospitalization when a clinical diagnosis has not been made. Such information will be valuable since precautionary measures may be initiated for personal protection as well as the protection of medical students and associates. Metallic bodies such as bullets may be readily located in medicolegal cases. Fractures may be more easily demonstrated radiographically than by dissection at the time of autopsy.

FIG. G. White male, 10 years of age. A. P. Chest, 6 foot distance, 60 Kvp, 50 Ma., ¼ sec.

In the training of X-ray technicians there will be an opportunity to demonstrate on the film specific organs in situ. In the training of residents in Radiology nothing can replace the opportunity to compare the radiographs with specific pathologic lesions. At Clinical Pathologic Conferences the radiologist will not be able to resort to the time-honored statement . . . "that occurred after we made the film." Furthermore, the pathologist will have to be more careful in his examination in order to satisfactorily explain the shadows that occur in the films. All in all a better grade of medicine can result in any institution from a closer co-operation between the radiologist and pathologist through the utilization of the opportunities made available by this X-ray-necropsy table.

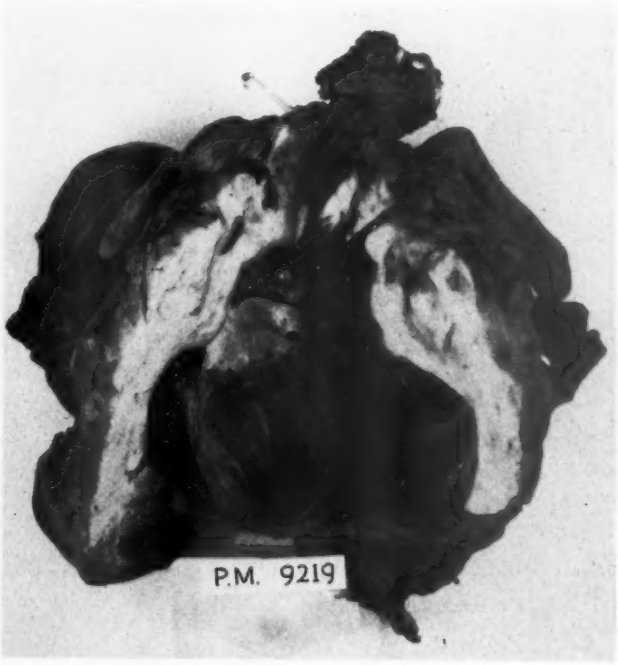

FIG. H. Gross specimen in figure G. Lymphosarcoma involving the mediastinum.

Summary

A combination X-ray-necropsy table is described. This consists of a 1/16 pure aluminum pan placed over the top of a conventional X-ray table. This combination table retains all the individual characteristics of an X-ray table and a necropsy table.

FIG. I. White female, 80 years of age. A. P. Chest, 6 foot distance, 60 Kvp, 50 Ma., ¼ sec.

FIG. J. White female, 80 years of age. A. P. Pelvis, 36 inch distance, 64 Kvp, 30 Ma., 2 sec.

FIG. K. White female, 80 years of age. Lateral lumbar spine, 36 inch distance, 80 Kvp, 30 Ma., 7½ sec.

USE OF CHORIO-ALLANTOIC MEMBRANE CULTURE SMALLPOX VACCINE IN TEXAS*

E. B. M. Cook, Bryan Bell, Philip Forsyth, J. V. Irons, and George W. Cox

Long before we became intimately acquainted with the causative agents of viral and rickettsial diseases, attempts had been made to gain protection against infection by the inoculation of materials from mild cases of diseases back into healthy individuals. For example, Jenner's use of cowpox pustular material to immunize against smallpox in 1796 and Pasteur's use of rabbit fixed rabies virus to prevent the develop of rabies in 1884, were applications of what we now know to be sound immunological technic; namely, that passage of a virus through a tissue medium foreign to its natural habitat may cause attenuation or destruction of its pathogenicity for man, while preserving its full immunizing capacities.

During the past fifty years some 59 human and 62 animal diseases of viral and rickettsial origin have been recognized and studied. We now have preventative vaccines for about 15 of the human diseases, prophylactic serums for 2, and preventative vaccines for about 10 of the 62 animal diseases. It is interesting to note that only three or four vaccines on this list are prepared by cultivation of the agent on tissue other than that of the chick embryo. In working with chick embryo vaccines it was found that individual tissue affinity, and consequently higher concentration of the infective agent, governs the choice of the embryonic tissue used for vaccine material. For example, with yellow fever and rabies vaccines the whole embryo is used; with typhus, psittacosis and Q fever vaccines the yolk sac; and with influenza and mumps vaccines the allantoic fluid. In preparing canine distemper, equine encephalomyelitis, fowl pox, laryngotracheitis, louping ill, and Newcastle disease vaccines, the embryo, the chorio-allantois, and the allantoic fluid are all

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used, while with pigeon pox and vaccinia vaccines only the chorio-allantoic membrane is used.

We felt that it would be of interest here to consider production and use of chick membrane smallpox vaccine since it is one of these tissue vaccines, not in common use except here in Texas.

Since we have only a small biological production division and felt that manufacture of smallpox calf lymph vaccine was outside our means and facilities, we were interested in the report of Goodpasture and Buddingh in 1935 on smallpox vaccine production using the chorio-allantoic membrane of the chick embryo. In 1939, after some preliminary experimentation, we undertook production by this new method.

Our first problem was the choice of a vaccinia strain to be used. We soon found that any calf lymph dermal strain of vaccinia would serve as a source of inoculum for the membrane cultures. Buddingh had shown that the dermal strain of vaccinia virus could be carried without intervening mammalian passage through 240 transfers in chorio-allantoic tissue, and that in over a 9-year period of cultivation in such tissue the virus was not appreciably altered in its pathogenicity and antigenicity for man. Our earlier studies on this same strain, carried on through the 309th passage, showed a reduction in titratable potency of the virus, and led us to the use of fresh calf lymph virus isolates for production of the seed virus. By the second to the fifth passage the virus titratable potency reaches its peak (Stearns, Wyatt, and Cook). Buddingh and Randall, in 1951, re-emphasized the fact that by the use of antibiotics and by careful inspection of the infected membrane, vaccinia virus could be freed of its calf lymph bacterial contaminants in one passage on the chorio-allantoic membrane. We found that the bacterial-free cultures so obtained had a vaccinia virus titer of 10^{-8} or higher by rabbit intradermal titration. The secret of maintaining this extremely high titer was simply the use of a fairly heavy virus inoculum dose (1:500 or 1:1000 dilution of tissue material) and by careful selection of the infected membranes harvested for vaccine use.

For mass production of vaccine we have modified the procedures originally outlined by Goodpasture and Buddingh for

inoculation and harvesting of membranes to a more simple technic. This technic is as follows:

The eggs to be used are purchased from a healthy, reliable source and incubated for 12 days, or the live 12-day old embryos may be purchased from a certified hatchery. The natural air sac and the position of the embryo are located by candling, marked, and the respective shell areas disinfected with a surface-acting germicide. Small holes are drilled in the egg shell at these two positions. With a sharp dissecting needle a small tear is made in the white shell membrane through the shell opening above the embryo. An artificial air sac is then created by applying gentle suction at the opening over the natural air sac. Then, using a small syringe and needle, the virus is inoculated through the artificial air sac opening. It is important that the inoculum be deposited on the chorio-allantois directly over the embryo. Both shell openings are then sealed with sterile paraffin. Uniformly large lesions are obtained by tilting or rocking the egg back and forth to spread the inoculum over the membrane.

The incubation period is 48 hours at 37° C. For the harvesting process the eggs are immersed in Roccal solution for 5 minutes to disinfect the shells. They are then opened by inserting a pair of sharp pointed scissors into the hole at the natural air sac opening and cutting through the shell, shell membrane, and the chorio-allantois. The excised top portion of the shell contains the infected lesion which is then picked off and placed in a sterile container and sterility tested. Membrane lesions so harvested are stored at minus 20° C. until made up into vaccine.

In making up the final vaccine, sterile membranes are pooled and emulsified in a tissue blender using whole inactivated beef serum as a diluent. Our search for a better diluent has resulted in the choice of beef serum instead of the traditional fifty per cent glycerine diluent so long used for smallpox vaccine.

The potency testing of the chick membrane vaccine is done by the method of Force and Leake. When applied to the scarified skin of a rabbit the membrane culture smallpox vaccine yields 80 to 100 per cent confluency of lesions in a 1:100 dilution, and a few discrete lesions in a 1:10,000 dilution. Viral content titers by the rabbit intradermal titration method are positive up through a 10^{-7} dilution.

After sterility and potency tests are completed the smallpox vaccine is distributed in sealed glass capillary tubes in the usual manner. Sterile puncture needles are supplied. The multiple pressure method of vaccination is recommended.

In view of the report presented at this Conference by Delaplane and Grumbles on the pleuropneumonia group of organisms occurring in chicken eggs as possible contaminants in embryos used for virus vaccines, we might comment more fully on our method of sterility testing of the chick membrane smallpox

vaccine. The culture medium used in checking the sterility of the membrane lesions, the bulk vaccine, and the final product is an excellent medium for the detection of bacterial contaminants (Vera), and is also recommended for the isolation, cultivation, and identification of the pleuropneumonia group. This fluid thioglycollate sterility broth contains cystine, trypticase, yeast extract, dextrose, and a pH indicator. The tests are read for any evidence of cloudiness or sediment and for pH change. Although we have not done animal inoculations into turkeys with this vaccine, we do feel that from the standpoint of sterility testing in this medium we are reasonably assured of a final vaccine free of such possible pleuropneumonia group contaminants.

After a two-year study of the use of the vaccine on a limited basis, we found it to be satisfactory in every respect and entirely comparable to calf lymph smallpox vaccine. It was then made available to all city and county health officers in the state. Report sheets were sent out with each shipment of vaccine but only a small percentage of the reports on use were returned. Sufficient reports were returned (7.5 per cent), however, to enable us to evaluate the vaccine used under field conditions. A summary of the field reports is presented in Table 1.

TABLE 1

Field Reports on the Use of Chick Membrane Smallpox Vaccine in Texas

Type Reaction	Persons Previously Vaccinated		Non-vaccinated Persons		Total of both Groups	
	Number	Per cent	Number	Per cent	Number	Per cent
Primary take	9,413	32.50	98,767	80.33	108,380	71.23
Accelerated take	7,346	25.36	4,474	3.63	11,820	7.77
Immune reaction	8,447	29.60	5,549	4.5	13,996	9.20
Total reactions	25,206	87.04	108,990	88.47	134,196	88.19
No. takes	3,753	12.96	14,203	11.53	17,956	11.80
Total reported	28,959	....	123,193	....	152,152	....

Total vaccine distributed (1939 through July 1952)—2,028,668 immunizations.
Total reports made—152,152 or 7.5 per cent.
Total positive reactions—134,196 or 88.19 per cent.

The reporting is purely voluntary and consequently very meager, but there are reports returned on each lot of vaccine distributed. We feel that such random reporting of field use,

in over 290 different towns, gives an encouraging picture of the efficacy of the chick membrane vaccine. While the total positive reactions in this field study were only 88.19 per cent, we have on several occasions obtained 100 per cent "takes" in small controlled non-vaccinated groups. While no one disputes the value of controlled studies in the evaluation of a vaccine, more information on just how a product stands up under conditions of field use is certainly desirable. It is to be regretted that we have no comparable studies on the field use of calf lymph smallpox vaccine.

In an evaluation of the reactions reported for the non-vaccinated group of persons, we had quite a few "immune reactions" listed (4.5 per cent). Possible explanations of these reactions are (a) that the person had had an undiagnosed case of smallpox, or that (b) a previous vaccination scar was missed, (c) vaccine of diminished potency was used, or (d) the vaccine was improperly administered. There has been a gradual decline of reported smallpox cases in both the United States and in Texas over the past twenty years. For example, in 1930 there were over 2,000 reported cases in Texas and in 1952 only one case was reported. We feel that preventative vaccination has undoubtedly played a role in this decline in Texas, as over the past thirteen years we have distributed over two million immunizations for smallpox, most of which were used in children. Other factors which might influence this decline in incidence are the undiagnosed or subclinical cases of smallpox which would result in a certain percentage of the general population being immune to the disease. The pattern of reported cases of smallpox in Texas for the past few years has been very sporadic with cases occurring in 38 different counties, usually with no traceable source case. This might indicate that the disease exists at a low level of endemicity with only an occasional case being clinically recognized. There is also the possibility that mild smallpox or alastrim is present, although we have had no reports of alastrim, all reported cases having been diagnosed as smallpox by the attending physician. Apparently cowpox cross immunity is not too important as a contributing factor in these immune reactions since it is presumed to be very rare in the United States. The few outbreaks reported in the literature have

occurred in dairy herds and have been traced to contact with human vaccination lesions. In Texas we have very little information on the prevalence of cowpox. Recently an outbreak has been reported in a dairy herd near Fort Worth, Texas.

We have been primarily concerned with the incidence of "no takes" reported. Poor vaccination technic can often condemn a vaccine unjustly. This was strikingly illustrated in a report by Orth and Robertson on a class exercise on immunization procedures at the Medical Field Service School, Fort Sam Houston, Texas. Using supposedly good technic, 595 class- member physicians vaccinated each other. There were 231 or 38.82 per cent "no takes" in this group, but subsequent revaccination, using a more careful technic, resulted in the reduction of these "no takes" to one per cent. A similar report of Benenson, Kempe, and Wheeler further stresses the importance of the technic of inoculating the vaccine. In a medical school class thoroughly drilled by assigned study, demonstration, and dry-run application, there were 22 per cent "no takes," while subsequent revaccination reduced this number to zero per cent. Judging from these results in controlled groups where proper technic of vaccination was being emphasized, perhaps our incidence of 11.8 per cent "no takes" in field use is not too surprising.

Of course, the efficacy of any vaccination process is based on protection of the immunized individual upon subsequent exposure to the disease. For the past 13 years in Texas, upon the report of any case of smallpox, mass immunization programs have been instigated in the community. Secondary cases of the disease have been virtually non-existent. The last major outbreak of smallpox occurred in 1949 when eight cases with one fatality were reported from Cameron and Hidalgo counties along the Mexican border. The date of onset of symptoms of the first diagnosed case was February 17, 1949. Health officials instituted a mass immunization program beginning about March 1st. Approximately 106,000 persons were vaccinated in Hidalgo County, 103,000 in Cameron County, and 30,000 in Starr County. Much of the vaccination was done in a five-day period between March 13th and March 18th. More than 99 per

cent of the vaccine used was chick membrane smallpox vaccine furnished by the State Health Department.

Of the eight recognized cases of smallpox in this outbreak, only one person had been vaccinated (12 years previously) and his attack was relatively mild. One of the children, who had a laboratory confirmed case, had twice missed the school vaccination clinics. His two older brothers had been vaccinated with chick membrane vaccine several years previously during routine school immunization programs. This child developed smallpox, and his two brothers escaped infection despite heavy familial exposure.

Our conclusions concerning the preparation and use of this chick membrane smallpox vaccine are:

1. The method is entirely suitable for production of a safe, highly potent vaccine.

2. It is an economical process requiring only a minimum of space and personnel. Each infected chick membrane furnishes enough vaccine for sixty to seventy smallpox immunizations.

3. Under conditions of field use this vaccine gives 88 per cent takes, and in carefully controlled experimental groups gives 100 per cent takes in non-vaccinated persons. It has been successfully used in controlling an outbreak of smallpox in Texas.

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CONTAGIOUS ECTHYMA IN MAN

D. A. PRICE*

Contagious ecthyma is a virus disease of sheep and goats which occurs in many parts of the world and is commonly called "soremouth" in this country. It is characterized by the formation of skin lesions (usually confined to the lips) which progress through the classical stages of papule, vesicle, pustule, and scab, with marked coalescence occurring subsequent to rupture of the pustules. Ordinarily, the only tissues affected are those parts of the skin which are practically devoid of fleece or hair; however, several observers have reported finding lesions on the buccal membranes. Before vaccination of susceptible animals became possible, the disease in Texas had reached major economic importance and even threatened to eliminate sheep raising as an industry, the gravity of the situation being due to infestation of lesions by screwworms (larval stage of the fly, *Cochliomyia americana*). In other states, (*e.g.*, Colorado) secondary infection due to *Actinomyces necrophorus* is reported to be responsible for most of the losses.

Natural Occurrence of the Disease. The disease is primarily an infection of lambs and kids, although mature sheep and goats are sometimes affected. At first, the lips are swollen and hyperemic for several days. Then papules become apparent upon close observation, and these gradually change to vesicles, then pustules, and finally scabs. It is characteristic that the pustules rupture and coalesce extensively, setting the stage for the formation of a continuous scab over the entire area. The scab tends to become quite thick, commonly in excess of one-half inch, and is deeply fissured and cracked. In the course of about three weeks, the scabs gradually fall off, and the lesion heals without a scar. If the scabs are removed forcibly, considerable bleeding occurs, the area is left quite raw, and a new scab forms.

It will be readily appreciated that lips so affected are quite stiff, unpliable, and perhaps painful. At any rate, there is some

* From the Texas Agricultural Experiment Station, Substation No. 14, Sonora, Texas. This report was given at the Third Annual Conference on Diseases in Nature Transmissible to Man, at Austin, Texas, April 13–15, 1953.

interference with suckling or eating and the animals suffer considerable loss of weight. Of great significance is the infestation of the lesions by screwworms.

FIG. I. Contagious ecthyma infection in man. Single lesion on upper lip.

The causative agent is contained in the scabs in plentiful supply and accounts for the repeated contamination of pastures and ranges. The virus is quite resistant to soil and weather conditions and retains its infectivity indefinitely.

Prophylaxis. A live-virus type of vaccine is the only product available for prevention of the disease, and annual vaccination of lambs and kids is commonly practiced on those Texas premises where contagious ecthyma is known to have occurred. Stated simply, the vaccine is prepared by artificially inoculating lambs, harvesting the scabs, reducing same to a very fine powder and suspending the powder in a suitable solution. Vaccination is accomplished in a manner similar to that used for smallpox in man. Although the actual disease is produced through vaccination, it is accomplished early in the spring before screwworm flies become active, and at a location of the operator's choosing, usually the inguinal area. Immunity probably exceeds two years in most cases.

The Infection in Man. The purpose of this paper is to relate the accidental infection of a laboratory worker with the virus

of contagious ecthyma, and to add an interesting note on a similar case reported by the author previously (Price, 1952).

Case No. 1. As previously reported, the author became infected while he was actively engaged in the preparation of contagious ecthyma vaccine at the Sonora laboratory. Duration and lesions closely paralleled the disease in sheep but were fortunately located on the wrist. The corresponding axillary lymph gland was moderately swollen and painful. When the lesions had reached the pustular stage, a small amount of the material was used to inoculate a lamb, wherein a typical vaccination reaction resulted. Material from this lesion, in turn, was infective for other lambs, and all lambs involved were subsequently shown to have developed immunity. The additional observation to be added here is that deliberate inoculation of the writer one year later failed to produce any reaction. Immunity was therefore presumed.

FIG. II. Contagious ecthyma in lambs.

Case No. 2. Another accidental infection of man was suspected when a laboratory assistant, also engaged in vaccine preparation, experienced a swollen upper lip, painful upon slight pressure. As the vesicular and pustular stages were reached, painful swelling of cervical lymph glands was noted. As in Case No. 1, material from the lesion was used successfully to infect a susceptible lamb. A scar remained after healing and has remained noticeable one year later due to the apparent destruction of hair follicles. Reinoculation to ascertain immunity was refused.

The incidence of human infection with this virus is not known, but if laboratory workers are so readily infected, then many persons in the sheepraising areas could be affected similarly. Although vaccine labels carry a warning regarding the

This photograph shows lesion on wrist of man (after scab had been shed) and scab-stage lesion at inguinal area of lamb, which represents transfer from man to lamb.
Photograph published in *The Southwestern Veterinarian*, Vol. 5, No. 4, 1952.

protection of the hands, most users pay little or no heed to the warning. Treatment of infected animals is often attempted and this, too, brings the human skin into direct contact with the virus. It would be interesting to know whether or not a serological or immunological relationship exists between this disease as it occurs in man and the dermatropic diseases more commonly found in man.

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ANTHRAX IN ANIMALS AND ITS RELATIONSHIP TO THE DISEASE IN MAN

C. D. STEIN*

This discussion will deal chiefly with the incidence, diagnosis, and control of anthrax in livestock and its transmission to man. The anthrax problem has been the subject of long and serious study in many parts of the world. Although considerable knowledge has been gained in combating the disease, many points in connection with the biology of the organism, both in nature and in the animal body, still remain unsolved.

Historically anthrax has a triple significance. It was the first disease of man and animals shown to be caused by a microorganism, the first disease against which a bacterial vaccine was found to be effective, and it constituted the principal subject for study by the early investigators who laid the groundwork for the modern science of bacteriology. Although anthrax of livestock has been a problem in the United States for more than 60 years and is one of the most serious diseases with which livestock sanitary officials have to deal, it has received very little attention in the veterinary literature in recent years.

Probably no disease of livestock causes more consternation and apprehension than anthrax when it first appears in a new area. This is not surprising when one considers the suddenness with which it strikes, its rapid spread, the heavy toll it takes and the danger of its transmission to man. The wide publicity sometimes given by the press to the occurrence of the disease in a heretofore anthrax-free area, also adds to the general state of alarm.

Anthrax exists in certain areas on all the continents and occurs from the tropics to the polar regions. The world-wide distribution of the disease and the difficulty encountered in combating it is due to the marvelous tenacity of the anthrax spore. Anthrax is spread from one country to another principally

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through infected animals and the interchange of contaminated objects closely associated with animal life, such as hides, hair, wool, bone meal, fertilizer, forage, and other materials. When anthrax is once established in an area, it may spread to adjoining localities and even to distant points by (1) contamination of soil, drinking water, and pasture plants with discharges of diseased animals; (2) by dogs, coyotes, and other carnivora; (3) by carrion-eating animals and birds, especially buzzards; (4) by flies and possibly other types of insects; (5) by streams contaminated with surface drainage from anthrax-infected soil, and tannery wastes; and (6) by contaminated food.

History and Incidence of Anthrax in Livestock. The early history of anthrax in livestock in the United States is somewhat obscure. The presumption is that it was implanted in the Rio Grande Valley and Mississippi Delta by explorers and primitive settlers from the Old World. In Louisiana, it has been traced back to the time of its settlement by the French. Widespread outbreaks in livestock and cases in man are recorded as early as 1835, and later in 1851 and 1884. The first cases in man recorded in the United States occurred in 1834 during outbreaks in livestock in Pennsylvania in the vicinity of Philadelphia. Outbreaks in Mississippi are reported to have occurred as early as 1836, and later in 1865. In Texas outbreaks are recorded as early as 1860, and later in 1880. Outbreaks in New York were recorded in 1881; in Vermont and Massachusetts in 1887, and in California in 1888. Infected areas still exist in most of these states.

The anthrax situation in livestock in the United States from 1915 to 1944, as determined by Bureau surveys, shows a gradual increase in territory involved. Outbreaks in livestock during that 30-year period were reported from at least 43 states, involving a total of 438 counties. The five states that reported having no outbreaks during that period were Arizona, Indiana, Maine, Michigan, and West Virginia.

During the past two decades one or more widespread outbreaks of virulent nature occurred in South Dakota, Nebraska, Mississippi, Louisiana, Texas, and California, while sharp outbreaks of a less severe nature occurred in Arkansas, Alabama, New Mexico, Missouri, and Nevada. Numerous sporadic out-

breaks occurred in other states. Recognized areas of infection of large dimensions exist in South Dakota, Nebraska, Arkansas, Mississippi, Louisiana, Texas, and California, while small areas exist in Vermont, Missouri, New Jersey, Delaware, Wisconsin, Utah, Nevada, and Oregon.

During the 7-year period 1945 through 1951, there were 1,141 anthrax outbreaks reported from 35 states with losses of 11,257 head of livestock. Outbreaks in new areas were reported from 133 counties in 27 states. During this period, 29 cases of agricultural anthrax were reported in man, 8 of which were in veterinarians.

Results of a nation-wide survey to determine the incidence of anthrax in livestock in 1952 indicate that 1,644 (anthrax) outbreaks in livestock were reported from 32 states involving 432 counties with a total loss of 3,451 animals. This is the greatest number of outbreaks ever reported during a single year. The tremendous increase in outbreaks was due principally to an unprecedented number of outbreaks that occurred in swine herds in the midwest and to the large number of outbreaks in Kansas, New Jersey, and other states, following prophylactic vaccination with certain lots of anthrax bacterin.

Outstanding features of the prevalence and distribution of anthrax in 1952 were its low incidence in recognized anthrax districts and the occurrence of numerous outbreaks with a low mortality rate in areas that heretofore have reported little or no anthrax. During 1952, Wisconsin reported four outbreaks in minkeries with total loss of 455 animals; New Jersey, one outbreak with a loss of 11 animals; and New York, two outbreaks with a loss of 206 animals. Records of the Federal Meat Inspection Division for 1952 indicate that at establishments operating under Federal Meat Inspection 195 hogs and no cattle were condemned for anthrax.

During the first six months of 1952, 738 outbreaks of anthrax in swine herds were reported from the states of Illinois, Indiana, Iowa, Michigan, and Ohio. The available history and circumstantial evidence in most instances indicated the infection in swine in the Midwest was of food origin.

A considerable number of the early outbreaks in swine in Ohio were traced directly to the use of mixed feed containing

raw bone meal imported from Belgium from which the organism of *B. anthracis* were isolated on laboratory examination. The outbreaks in swine were characterized by the following unusual aspects: (1) wide distribution; (2) occurrence during the winter in noninfected areas; (3) low morbidity and mortality rate; (4) failure to spread from one premise to another; and (5) failure to spread by contact from animal to animal or from swine to other species.

During the 5-month period from May to September, 1952, inclusive, a total of 419 outbreaks involving the loss of 507 head of livestock were reported in "bacterin" vaccinated animals from Kansas, New Jersey, Florida, and New Mexico, most of which occurred in Kansas. The history associated with the post-vaccination outbreaks indicates that reactions occurred in a high percentage of the herds of cattle or flocks of sheep vaccinated with certain serials of anthrax bacterin.

History of Anthrax in Texas. Anthrax has been a problem in Texas for many decades. The low marshy land along the Gulf of Mexico is generally considered one of the oldest anthrax areas in the United States. According to Williams outbreaks were recorded at United States Army Posts in Texas as early as 1868. The disease was also reported near Fort Clark in 1880.

High incidence of anthrax was reported: (1) in 1904 in southeast Texas near the Louisiana line; (2) in 1908 in the low lands adjacent to the Gulf; and (3) in 1914 to 1916 in at least 39 counties. During the outbreaks in 1915 Dr. Christman, State Veterinarian of Texas, acquired the infection and died of the disease. In 1920 widespread cases of anthrax occurred in new areas in South Texas involving 9 counties. An outbreak that occurred at Fort Bliss in 1923 was believed to be due to hay brought in from infected areas.

In 1926 more than 2,500 head of livestock including cattle, horses, swine, sheep, and goats died of the disease in the Dry Frio Canyon area. Wild rabbits and squirrels were reported to have acquired the infection during this period.

In 1941 it was reported that Dr. Mack, a veterinarian of Bay City, Texas, developed anthrax following a fly bite on the forearm. During the same year anthrax involving several species of animals was reported at the Hermann Park Zoological

Gardens in Houston, Texas. The source of the outbreak was traced to feeding infected horsemeat.

In recent years covering the period from 1945 to 1953 inclusive, Texas has reported cases yearly. During that 8-year period a total of 265 outbreaks with losses of 1,559 head of livestock have been reported to the Bureau. During the first quarter of 1953 cases of anthrax were reported from four counties—Brazos, Brazoria, Blanco, and Hamilton.

Causative Agent. *Bacillus anthracis*, which is the specific cause of anthrax, is a non-motile, gram-positive, spore-bearing rod possessing certain properties that make it one of the most unique pathogenic microorganisms encountered in nature. In certain areas, especially those subject to periodic flooding, in low-lying marshy land, or in soils that are rich in decomposed vegetable or animal remains, the anthrax organism survives for long periods. The organisms possess a high degree of virulence and when they gain access to the animal body, they multiply rapidly, invade the blood stream, and produce septicomia.

When exposed to adverse conditions in the presence of oxygen, the bacilli forms spores which, on account of their remarkable tenacity, are of great hygienic importance. They are very resistant to heat, low temperature, chemical disinfectants, and prolonged drying. The bacilli when suitably stained show capsules in the blood smears made from animals dead of the disease, but no sporulation occurs in the unopened carcass.

Numerous instances are on record to show that spores retain their viability in the soil, in water, on hides, and in storage for many years. Evidence has been reported to show that they may survive and retain their virulence in the soil for at least 25 to 30 years and that dry anthrax spores may survive at room temperature for more than 40 years.

Animals Susceptible. Virtually all animals are susceptible in some degree to anthrax. Cattle, horses, sheep, goats, and the wild herbivores are most commonly affected. Man and swine possess a greater natural resistance to the disease. Under certain conditions, dogs, cats, and wild animals of prey, birds, frogs, and toads may become infected. Mice, guinea pigs, and rabbits, which are commonly used in the laboratory diagnosis of anthrax, are very susceptible, while rats are less so.

Infection in cattle, horses, mules, sheep, and goats, usually is the result of grazing on infected pasture land. Infection may also be caused by contaminated fodder or artificial feedstuffs, such as bone meal, blood meal, oil cake, and tankage; by drinking from contaminated pools; or by the bites of contaminated flies. Dogs, cats, and other carnivora usually acquire the infection from consumption of infected meat.

In the United States anthrax occurs in epizootic form in regions in which the soil is known to be seriously infected. However, it may occur sporadically anywhere at any time and thus may appear where previously not identified or where it has been quiescent for a long period.

In areas commonly designated as "anthrax districts" it constitutes a perennial problem, making its appearance during a definite period, known as the "anthrax season," usually late summer and early fall when grazing is closer due to scanty pasturage and when flies are very numerous. In such areas the disease can be kept in check by preseasonal vaccination with appropriate types of immunizing agents.

Symptoms and Lesions. Since symptoms and lesions are important in making a clinical diagnosis, they will be briefly discussed. Anthrax may occur in a peracute, acute, subacute, or chronic form. The peracute form is most common in cattle, sheep, and goats, occurring at the beginning of an outbreak. It is characterized by sudden onset and a rapidly fatal course. Victims are frequently found dead without showing any previous evidence of disease.

In the acute and subacute forms there is first excitement and a rise in body temperature, followed by depression, spasms, respiratory or cardiac distress, trembling, staggering, convulsions, and death. During the course of the disease pregnant animals may abort, rumination ceases, and the milk secretion is reduced. Bloody discharges may emanate from the natural body openings and edematous swellings may appear on different parts of the body. The acute form usually terminates in death in a day or two, while the subacute form may lead to death in three to five days or longer, or to complete recovery after several days. These types of the disease are common in cattle, horses, and sheep.

A cutaneous form of anthrax characterized by swellings in various parts of the body may occur in cattle and horses following vaccination or following bites by infected flies. Chronic anthrax occurs occasionally in cattle, horses, and dogs with local lesions confined to the tongue and throat, but is observed mostly in swine, which are more resistant to the disease than any other domesticated farm animal.

Carcasses of animals dead of anthrax decompose rapidly and soon become greatly bloated. Rigor mortis is either absent or incomplete. The blood is considerably darker than normal and does not clot readily. Hemorrhages beneath the skin are common. Clear or somewhat blood-tinged gelatinous exudates are found between the muscles and beneath the skin, especially in the areas where swellings were seen before death. The spleen in acute cases usually shows characteristic changes, which are of considerable assistance in making a diagnosis of anthrax. This organ is greatly enlarged, and the splenic pulp is dark red to blackish in color and soft or even semifluid in consistency. The liver, kidneys, and lymph glands are usually congested and enlarged and show areas of hemorrhage.

Diagnosis. Areas used for pasture in known anthrax districts should be under close observation for evidence of the disease. Anthrax should be suspected when animals die on or near premises where the disease has appeared previously. Final and conclusive diagnosis is based on laboratory examination, which entails a grave responsibility and should be performed with care.

While some laboratory workers consider that anthrax can be diagnosed easily and quickly by microscopic examination, experience has shown that it is hazardous to base a diagnosis entirely upon examination of blood smears. Many organisms, particularly those found in specimens undergoing putrefaction, may be confused morphologically with *B. anthracis* and, even though the microscopic picture appears conclusive, it should always be checked by both cultural and laboratory animal inoculation tests to guard against possible error in diagnosis.

It should be pointed out that cultural tests for anthrax on specimens or material contaminated with (1) rapidly growing organisms of the spreader type, (2) organisms especially an-

tagonistic to anthrax, or (3) anthrax-like organisms may frequently miscarry.

Control Measures. The effective control of anthrax in livestock requires the combined action of livestock sanitary officials, local veterinarians, and owners of livestock. When a diagnosis of anthrax has been made, the following measures are generally recognized as the most effective means of control.

(1) The prompt and proper disposal either by complete burning or by deep burial of animals dead of the disease, together with all the manure, bedding, blood-stained soil, and other contaminated material.

(2) A careful examination of the herd for animals showing early symptoms of the disease; the prompt isolation of sick animals, and immediate treatment with large doses of anti-anthrax serum and/or penicillin.

(3) Vaccination of the apparently well animals in the herd as soon as possible, for prevention, in accordance with methods recommended by the State Livestock Sanitary officials and other experienced veterinarians.

(4) Immediate change of pastures if possible. This precaution in itself has in many instances helped to reduce losses.

(5) A strict quarantine of premises rigidly enforced to prohibit the movement of livestock or other commodities from or into the infected area.

(6) Good sanitation and fly control are also important.

(7) Due precautions should be taken to prevent spreading the infection through rats, dogs, cats, hogs, chickens, buzzards, crows, and other animals that feed on the carcass of an animal dead of the disease.

Vaccination. Anthrax is one of the few serious diseases of livestock that can be largely controlled by preventive vaccination. A material reduction in the occurrence of the disease can be accomplished by annual vaccination of all stock in infected localities well in advance of the anthrax season.

The recognized immunizing agents now being used in the United States for vaccination of animals against anthrax are of two types—sterile products (anti-anthrax serum and anthrax bacterin) and the living-spore vaccines which consist of sus-

pensions of living anthrax spores of different degrees of attenuation suspended in a solution containing normal saline in combination with glycerine, saponin, or alum.

Anti-anthrax serum is of value both as a preventive and as a therapeutic agent. Experience has shown that living-spore vaccines produce a higher degree of immunity than do bacterins.

Experimental evidence to confirm this fact was obtained by Bureau investigators in immunity tests carried out with sheep. While satisfactory results have been obtained with spore vaccines given subcutaneously, very good results following the use of intradermic spore vaccine have been reported by investigators and veterinary practitioners, both in Europe and the United States. Reports from the field indicate that in recent years the intradermic method of vaccination (with anthrax-spore vaccine) is gradually growing in favor, particularly in badly infected anthrax districts, and that the results obtained from its use have been highly satisfactory. During the period from 1939 to 1951, thousands of cattle on five Indian reservations located in known anthrax districts in South Dakota, were vaccinated by the intradermic method under direct supervision of Bureau of Animal Industry veterinarians, with excellent results.

Treatment in Animals. For many years homologous anti-anthrax serum was most commonly used for the treatment of the disease in animals. Intravenous injections of the serum in large doses of 50 to 100 cc. or more during the early stages of the disease gave the best results.

Arsenicals and sulfa drugs have been used only to a very limited extent in treating animals, but penicillin, which has been used in human anthrax with excellent results, is now being used with equally good results in the treatment of infected animals. Excellent results in the treatment of infected livestock by penicillin therapy has been reported from different parts of the United States and France. In cattle, intramuscular administration of one to two million units or more of penicillin resulted in marked improvement in 36 hours or less with complete recovery in 4 to 5 days. Good results from treatment with penicillin combined with anthrax antiserum have been reported

from a number of the states, while Dr. Bailey, a veterinarian in New Jersey, reported excellent results following treatment of cases of vaccine anthrax with terramycin intravenously—2 grams daily for 3 days or longer.

Correlation Between Anthrax in Animals and Anthrax in Man. Although anthrax is considered to be primarily a disease of animals, it is transmissible to man, either directly by contact with infected animals or indirectly by contact with infected animal material or objects contaminated with the organism. Anthrax in man, therefore, is classified either as an occupational or non-occupational disease, depending upon the source of infection. Occupational anthrax is referred to as either of agricultural or industrial origin. Agricultural anthrax is acquired by close contact with infected animals or their carcasses, usually by handling, skinning, or making post-morten examination; while industrial anthrax is acquired by indirect contact, as in the manipulation of infected animal material, such as hides, wool, and hair. Non-occupational anthrax is acquired from the use of infected shaving brushes, wearing apparel, and other indirect sources. It is not encountered as frequently as occupational anthrax.

In man, anthrax usually occurs as a primary localized infection of the skin in the form of a carbuncle—or as an infection of the lungs, which is known as "wool-sorters' disease." In countries where the flesh of animals dead of disease is eaten, an intestinal form of anthrax has been reported. Skin infections result from the handling of carcasses of animals dead of anthrax or the hides, hair, or wool from such carcasses. During outbreaks in animals, cases in man have been repeatedly reported from fly bites. Originating as localized infections in the form of small pimples, the lesions develop rapidly and may terminate in a fatal septicemia or blood poisoning. Prompt medical attention is most important whenever anthrax infection is suspected. The pulmonary form of the disease results from the inhalation of anthrax spores in factories where hair and wool are processed. This form of anthrax runs a very rapid course and usually terminates fatally. Anthrax is occasionally transmitted to man by spore-infected shaving brushes, by wear-

ing apparel, such as furs and leather goods, or by other animal by-products not properly sterilized.

The U. S. Public Health Reports covering the 8-year period from 1944 to 1951, inclusive, show a total of 428 anthrax cases in man. These figures indicate that while human anthrax in the United States is not a common disease, it certainly cannot be considered a rare disease.

In the treatment of anthrax in man, promptness of diagnosis and early treatment are of first importance.

While specific anti-serum, arsenicals, and sulfa-drugs have given excellent results in treatment of anthrax in man during the past 20 years, the trend in recent years appears to be away from these preparations and toward penicillin which has been found to be highly effective. Very recently some of the newer antibiotics, such as aureomycin, chloromycetin, and terramycin have proved to be extremely effective in the treatment of a limited number of cases.

Relation of Anthrax to Food Hygiene. Public Health officials have stressed the importance of preventing the transmission of anthrax to man through the consumption of meat and other animal products.

Although the intestinal form of anthrax has been reported from countries where the flesh of animals dead of disease is consumed, there is little likelihood of infection being acquired from meat in civilized countries, where stringent meat inspection regulations are enforced. Some element of danger, however, may exist in the meat of animals slaughtered in uninspected abattoirs, or in meat used as animal food from local rendering plants.

The danger of infection through milk containing bacilli is remote, as the milk secretion usually diminishes greatly, and the bacilli, as a rule, are present in the blood only, in great numbers just before death and rarely occur in the milk secretion.

The question may arise as to whether the meat and milk from vaccinated animals are dangerous. So far as we know, no instance has been reported to indicate that milk from vaccinated animals contains the organisms. However, milk from cows showing a marked reaction following vaccination should not be used until animals have returned to normal.

Owing to the fact that viable anthrax spores may persist at the site of inoculation for a considerable period following vaccination, the Federal Meat Inspection Regulations require that carcasses of food-producing animals treated with spore vaccines be condemned if the animals are slaughtered *in less than six weeks* following vaccination, or if evidences of the reaction to the injection remain, such as inflammation, tumefaction, or edema, regardless of the period of time elapsing between treatment and inspection.

Livestock Sanitary Control. Livestock sanitary laws and regulations for the control of anthrax have been enacted in all highly civilized countries. In areas where the laws have been enforced, such as England, France, Germany, sections of South Africa, and certain localities in the United States, there has been a marked reduction in its prevalence both in animals and man.

The control of anthrax in the various states is carried out principally by the livestock sanitary officials, the practicing veterinarians, and livestock producers. However, in extreme emergencies when heavy losses from anthrax occur and when the combined forces of state and practicing veterinarians are inadequate to cope with the situation, the Bureau has endeavored to furnish what assistance was available.

The numerous widespread outbreaks of anthrax that occurred during 1952 caused great concern among livestock sanitary officials and public health officials and resulted in special action being taken to combat the disease and prevent its spread.

During the first quarter of 1952, members of the U. S. Livestock Sanitary Association representing 16 states met with officials of the U. S. Department of Agriculture, the U. S. Public Health Association, the Food and Drug Administration, and Civil Defense Administration to discuss ways and means of combating the spread of the disease. Several resolutions concerning the problem were passed at the meeting, which resulted in the adoption of the following measures designed to assist in the control of the disease:

1. The Bureau's regulations covering the importation of bone meal have been amended to prohibit the admission of

so-called raw bone meal for use in either fertilizer or feed. However, steamed bone meal or bone meal heated sufficiently to destroy anthrax spores is still permitted entry. All imported animal bones must be consigned to an approved establishment for further processing, the adequacy of the sterilizing equipment being determined by the Bureau.

2. Many of the states have likewise passed laws regulating the operation of rendering plants and feed mixing plants and requiring that all bone meal and other animal products used in fertilizer or feeds be treated sufficiently with heat to destroy anthrax spores.

3. The U. S. Public Health Service with the assistance of the U. S. Bureau of Animal Industry, the Federal Food and Drug Administration, and the Federal Civil Defense Administration have prepared a new set of specific regulations endorsed by the co-operating agencies in relation to anthrax in dairy herds, which have been recommended for inclusion in the Milk Ordinance and Code of the U. S. Public Health Service as applied to anthrax. These recommendations have been approved by the U. S. Livestock Sanitary Association and copies of the recommendations were sent on February 4, 1953, to all State and Territorial Milk Control Authorities and others concerned.

4. In order to have up-to-date information on the prevalence and distribution of the disease in livestock, arrangements have been made to have each state report monthly to the Bureau all outbreaks of anthrax occurring within the state so that the Bureau can in turn issue a report on the national incidence each month.

5. Special committees on Anthrax have been appointed by the U. S. Livestock Sanitary Association and the American Veterinary Medical Association to study the anthrax problem and make recommendations for its future control.

A REVIEW OF THE EFFECTS OF SELENIUM ON THE HEMATOPOIETIC SYSTEM*

MARY LEE WALKER AND R. H. RIGDON

The economic problems created by the poisoning of livestock by seleniferous grains have resulted in many experimental studies on various aspects of selenium poisoning. The effects of selenium on the hematopoietic system have been observed by a number of different workers. Trelease and Beath (66) in 1949 published a comprehensive survey of the selenium problem and included in it some of the hematological studies. Several other reviews have been published on various phases of the selenium problem (4, 17, 30, 37, 42, 44, 47, 54, 57, 63, 64, 65, 67, 72). It was thought that a more extensive review of the literature pertaining specifically to the hematological effects would be of value.

Animals are poisoned by selenium in either of the following ways: inhalation, ingestion or the experimental administration of small amounts of selenium compounds over long periods of time; or the inhalation, ingestion or experimental administration of selenium compounds in large quantities within short periods of time. The former is chronic poisoning and has been referred to as "alkali disease" in earlier publications. When the onset of symptoms is early and severe, it is acute poisoning. This form has been called "blind staggers" by some of the earlier workers in this field. The hematological changes in these two types of poisoning differ.

Historical Background. The first account of what is recognized today as selenium poisoning was published by Madison in 1860 (29). Apparently the first experimental observation on the blood in selenium poisoning was made on dogs by Rabuteau in 1869 (48). He observed crystals in the blood stream. Such crystals have not been found reported by other investigators. Modica (41), in 1897, made the first observation of anemia when he produced chronic poisoning in rabbits by the injection of selenium. In 1929, Franke (11) began the experi-

* From the Laboratory of Experimental Pathology, The University of Texas Medical Branch, Galveston, Texas.

ments which eventually led to the discovery in 1935 that selenium was the toxic agent in foliage plants that had been used for food by animals developing clinical symptoms of poisoning. Since the publication of Franke's observations (11), numerous experiments have been made with selenium using a variety of animals both in the laboratory and in the field. Apparently selenium is the only mineral element known to be absorbed by food foliage plants in sufficient quantities to render them lethal when consumed by animals (66). Both organic and inorganic selenium compounds have been used in the experimental study of selenium poisoning. These have been administered orally, cutaneously, subcutaneously, intraperitoneally, and intravenously. The toxic effects are influenced by the type of compound used and the mode of administration. Selenium compounds are distributed widely through the body by the circulatory system and accumulate in varying amounts in different tissue. High concentrations have been found in the blood, liver, kidney, hair, and hooves of horses chronically poisoned by small doses of sodium selenite (40). McConnell *et al.* (35) recently have determined the relative quantity of radioactive selenium in the different viscera of the duck. It is of interest to note in these studies that radioactive selenium was found within the bone marrow. These workers have also tagged duck erythrocytes and found the length of life of the red cell to be 11.7 days.

It is suggested that selenium produces its toxic effect through an interference with enzyme systems. The mechanism of this action has been discussed in a paper by Smith (57). He has suggested that the inhibition of oxygen consumption by tissues results from the poisoning of succinic dehydrogenase by selenium. Wright (73) was unable to demonstrate any impairment of the cytochrome-indophenol-oxidase but there did occur a general poisoning of the dehydrogenating enzymes. The observation that reduced glutathione could reactivate succinic dehydrogenase previously inactivated by oxidized glutathione suggests the possibility that the injurious effects of the selenium compounds are due to the removal of sulfhydryl groups that are essential for the oxidative mechanism (21). Liver arginase has been found to be unaffected by inorganic sele-

nium compounds *in vitro* and apparently it was augmented in some instances by the ingestion of seleniferous wheat (74).

Selenium is eliminated from the body by many routes; through the mammary glands, across the placenta, in the feces, bile, lungs, perspiration, but principally by the kidney (34, 66, 71).

Effects on the Erythrocyte. Selenium has been demonstrated in red blood cells following its administration in both the inorganic and organic form (31, 70). The fixation of selenium in the red blood cells has been reported by Dudley (5) and McConnell and Cooper (33). The latter found that 24 hours after the last of three injections of sodium selenate containing radioselenium the whole blood activity in red blood cells for four dogs averaged 79 per cent, while in plasma it averaged 22 per cent. It was further shown that selenium was present in crystalline hemoglobin and in hemin and in globin. The amount of selenium in hemin when calculated on the gram basis was greater than in globin. The fixation of selenium in hemoglobin of the rabbit and rat and in hemin of the duck has likewise been demonstrated (35).

The position of selenium in hemoglobin, as well as the mechanism by which it is fixed in hemoglobin, is not known. The incorporation of selenium in hemoglobin may take place by exchange with some normally occurring substance or by addition to an available radical after diffusion across the cell membrane.

As a working hypothesis it has been postulated (34) that the formation of fixed selenium in red blood cells (selenohemoglobin) is similar to that of sulfhemoglobin. The latter has been characterized as having an absorption band of 623–626 mμ (18). Michel (36) has shown that sulfhemoglobin contains one more sulfur atom than hemoglobin and has outlined the following reaction:

$$HbO_2 + H_2S \rightarrow Hb + S + H_2O_2$$
$$Hb + H_2S + H_2O_2 \rightarrow HbS + 2H_2O$$

Selenohemoglobin, like sulfhemoglobin, as shown by Clarke and Hurtley (3), can be produced by passing H_2Se into fresh blood which has an absorption band at 613–628 mμ. The formation

of selenohemoglobin *in vivo* by this mechanism appears to be possible for it is well known that the animal organism has the ability to convert inorganic selenium into a volatile form which is subsequently exhaled in the respiratory gases (20, 55, 32).

Available information about the constitution of selenohemoglobin appears to be limited with information on sulfhemoglobin predominant. As speculated from differences in the absorption spectrum, it has been reported that the chief difference between sulfhemoglobin and oxyhemoglobin may be in the hemin group (24). This idea is more or less substantiated by Michel (36) who demonstrated electrophoretically that the general character of the protein in sulfhemoglobin and oxyhemoglobin is the same.

It is likely that the porphyrin ring in sulfhemoglobin is unbroken (28). Haurowitz (18, 19) suggested that two $-SO_3H$ groups are attached to two of the methane bridges of each porphyrin ring and that subsequent condensation takes place with the adjacent vinyl group. This type of bond, however, does not account for the strengthened linkage between the hemin and the protein part of the molecule that Haurowitz found (18, 19). This bond between protein and hemin might be the result of a condensation with loss of water between the $-SO_3H$ and a basic group of the protein globin.

The first observation on the effect of acute selenium poisoning on red blood cells apparently was made by Jones in 1909 and 1912 (22, 23). He observed that erythrocytes at first were normal both in number and hemoglobin content in the rat given sodium selenite; however, later these cells showed cloudy white centers and were vacuolated. Jones also made the observation that hemolysis did not occur *in vitro* but it did when sodium selenite was injected "hypodermically." Kondo (26, 27) using organic selenium compounds, observed hemolysis *in vitro*, but not *in vivo*. Miller and Williams (39) noted that in acute poisoning in horses the blood macroscopically was almost black in color, but there was no hemolysis. In this laboratory we have not observed any hemolysis in ducks given large amounts of sodium selenite either orally or subcutaneously.

Ellis *et al.* (9) produced an acute poisoning in the catfish and noted an anemia accompanied by many immature red

blood cells. Nucleated red cells have been found in the peripheral blood of rabbits on the second to third day following the intravenous injection of colloidal selenium (1). In these rabbits the erythrocyte count increased immediately following the injection, reaching its peak after approximately two hours. The number of red cells in the blood progressively decreased; however, in some rabbits it was considered to be elevated for as long as ten days. We also have observed a marked increase in the number of erythrocytes in the peripheral blood of some White Pekin ducks given sodium selenite subcutaneously (50). This we have considered to be the result of hemoconcentration since the birds were very thirsty and apparently consumed large amounts of water for several hours following the time of injection of the selenium. An anemia subsequently developed, however, in these ducks. Smith and associates (60) failed to note an anemia in rats with an acute or subacute selenium poisoning. The failure of an anemia to occur in Smith's experiments may have been influenced either by the compound used or the quantity given. We have produced an anemia in rats weighing 375 grams by the intraperitoneal injection of four doses of 1.0 cc. each of sodium selenite containing 0.75 mg. of selenium over a period of three days.

The most obvious hematological change observed in chronic selenium poisoning is the anemia. Franke and Potter (14, 15), while feeding toxic grain to rats, observed a distinct tendency toward anemia after the first nine to seventeen days. Moribund animals had red blood cell counts as low as 450,000 cells per cubic millimeter. Franke and Potter (14, 15) demonstrated that this anemia was not due to inanition, but suggested that it might be the result of one or more of the following factors: (1) Lack of blood formation; (2) Direct hemolysis by the selenium; or (3) Hyperactivity of the normal blood cell-destroying mechanisms. Rhian and Moxon (49) observed that the decrease in the number of red blood cells was accompanied by a proportionate decrease in the quantity of hemoglobin. They expressed the opinion that the anemia was due to subnormal formation of erythrocytes. Anemia in chronic selenium poisoning has been observed by Moxon (42) and Smith and co-workers

(56, 58, 59, 60). The anemia in the rat has been considered to be essentially microcytic and hypochromic (60).

An increase in the number of reticulocytes may accompany the anemia in chronic selenium poisoning. This may reach a peak as high as 82 per cent in well developed anemias (56, 60). Anisocytosis, poikilocytosis, and polychromatophilia were also present in rats with anemia resulting from chronic poisoning (14, 15, 56, 60). We have failed to observe any morphologic changes in the erythrocytes of ducks given selenium although there does occur an anemia. A decrease occurs in the number of erythroblasts in the peripheral blood of ducks during the first few days when given large amounts of sodium selenite. An increase occurs in the number of erythroblasts in the peripheral blood, however, when a small amount of the drug is given over a longer period of time (50).

Smith (56) noted that the quantity of protein in the diet affected the development of anemia in rats given selenium orally. Those fed a low protein-high carbohydrate diet developed an anemia while those fed a low protein-high fat diet showed no significant hematological changes. A high protein-low carbohydrate diet produced no effect either on the hematopoietic system or on the growth and reproduction of the rat. Rosenfeld and Beath (52) observed that sheep fed a medium-high protein diet were more resistant to the effects of selenium poisoning than sheep fed a low protein diet. From these observations, it may be concluded that a diet rich in protein may afford some protection against selenium poisoning.

Effects on Hemoglobin. The effect of selenium on the concentration of hemoglobin in the blood has been frequently reported. Investigators have observed a decrease in the quantity of hemoglobin in rats chronically poisoned (11, 12, 14, 15, 42, 49, 56, 60). Franke (11) reported that chronically poisoned rats develop a progressive anemia and may die with hemoglobin levels as low as 2.1 gms. per 100 cc. Franke and Potter (14, 15) observed that their rats had abnormally high levels of hemoglobin prior to the 9th to 17th day of the experiment; after which time, an anemia developed accompanied by a gradual decline in the amount of hemoglobin terminating in a sudden drop to fatal levels. These authors (15) suggested that,

"High levels of hemoglobin are probably a characteristic symptom of acute selenium poisoning. The absolute amount of hemoglobin is almost certainly not increased since the blood is probably anhydremic." Rhian and Moxon (49) noted that the decrease in hemoglobin in chronically poisoned dogs was proportionate to the decrease in the number of erythrocytes. They suggested that there was an actual decrease in the number of red blood cells rather than a decrease in hemoglobin content of the individual cell. Cattle grazing on a seleniferous range have shown a decreased hemoglobin level (44).

In acute selenium poisoning in dogs, an increase of as much as 62 per cent in hemoglobin values has been observed (2). These findings serve to substantiate the suggestion made by Franke and Potter (15) that high hemoglobin levels may be characteristic of acute selenium poisoning. Fish with a delayed reaction from acute selenium poisoning had hemoglobin levels of approximately 3 gms. per 100 cc. below normal values (normal: 9.8 gms. per 100 cc.); as this hemoglobin level is far out of proportion to the slight lowering of the red blood cells found, it suggests that the selenium was interfering with normal hemoglobin formation (9).

Effects on the Leucocyte. Franke and Potter (14, 15) noted a variation in the ratio of the leucocytes with a tendency toward leukopenia in rats during the terminal stages of chronic selenium poisoning. Jones (22, 23) observed a slight lymphocytosis followed by a more marked increase in the number of polymorphonuclear leucocytes in acute selenium poisoning in the rat, the cat, and the rabbit. Ellis *et al.* (9) found an increased number of white blood cells with a lymphocytosis of 82 per cent in fish with a delayed reaction to acute poisoning. Achard and Ramond (1) noted a leucocytosis in rabbits given a single intravenous injection of colloidal selenium. In some of the rabbits the increase was 1,500 cells, while in others the number was double the normal after an interval of two hours. Subsequently the leucocyte counts returned to normal levels. Duhamel (8) observed leukopenia in young rabbits two to three hours after receiving a similar preparation of selenium. It was suggested that the occurrence of either a leukopenia or leucocytosis in the rabbit was related to the age of the animal and the quantity

of the drug administered. The differential count apparently was not affected (1).

Effects of Selenium on Some of the Other Blood Elements. Anderson and Moxon (2) observed a marked increase in hematocrit values in acutely poisoned dogs. Such, of course, would be expected to accompany the hemoconcentration that has been observed in acute poisoning. Ellis *et al.* (9) noted a lowering of the specific gravity of the blood in selenium-poisoned fish showing a delayed type of reaction. Kondo (26, 27) did not find any macroscopic changes in the bone marrow of rabbits with subacute poisoning; however, microscopically there was hyperplasia in both the nucleated and non-nucleated red blood cells. Achard and Ramond (1) also observed hyperplasia of the rabbit's bone marrow after an intravenous injection of colloidal selenium. This hyperplasia in the rabbit began to decrease by the sixth day and by the tenth day the bone marrow had returned to normal. At first this hyperplasia resulted from an increase in the number of neutrophilic myelocytes. On the sixth experimental day the number of erythroblasts began to increase and the neutrophils began to decrease. These specific observations on the rabbit's bone marrow are interesting; however, the opinions are based upon only three rabbits. Smith *et al.* (60) reported that in chronic poisoning in the rat "bone marrow has always been found to show hypoplasia in greater or less degree." It is of interest to note this variation in the bone marrow in subacute and chronic selenium poisoning as reported by different investigators. It has been shown in this laboratory that acute selenium poisoning in the duck is accompanied by hypoplasia of the erythroid elements within the bone marrow (50).

Fimiani (10) observed an increase in prothrombin time in chronically poisoned rabbits. One might expect an increase since selenium injures the liver.

Selenium in the Blood. Selenium has been detected in the blood by a number of workers (5, 6, 7, 16, 25, 31, 38, 39, 40, 45, 46, 49, 51, 52, 53, 61, 62, 68, 69). Dudley (5) found selenium in the red corpuscles in a protein-like complex, but not in the plasma and fibrin. Smith and associates (61) noted that the erythrocytes contained more selenium than the plasma.

McConnell (31) injected rats subcutaneously with a subtoxic dose of sodium selenate containing radioselenium and observed that "Selenium first appears in the blood in greater amounts in the plasma than in the red blood cells and is removed from the plasma faster than from the red blood cells. It would appear from the data that the rate of deposition in red blood cells was more rapid than the rate of elimination, and that a certain amount of fixation of selenium occurs." Rosenfeld and Beath (53) studied the metabolism of sodium selenate and selenite by animal tissue *in vitro* and found that selenate was converted into selenite by various fresh tissues. The highest activity is shown by the beef liver followed by the spleen, whole blood and plasma. The red blood cells are unable to change selenate to selenite. The attachment of selenite to tissue proteins is evident from these results according to Rosenfeld and Beath (53).

Animals Used for Hematological Studies on Selenium Poisoning. A variety of animals has been used in the hematological research on selenium poisoning. These include, in decreasing order of frequency, the rat, rabbit, cat, cow, hog, sheep, dog, horse, fish, and duck. The rat has been used most frequently both because of its adaptability as a laboratory animal and because of its susceptibility to selenium (11, 12, 13, 14, 15, 22, 23, 25, 31, 42, 50, 54, 56, 58, 59, 60). The rabbit, although less susceptible to selenium than the rat, is also quite adaptable as a laboratory animal and has been used by several investigators (1, 6, 8, 10, 16, 22, 26, 27, 41, 58, 62, 68, 69). The cat, although quite resistant to selenium poisoning, has been used primarily for the purpose of the detection of selenium in the blood (22, 58, 60, 61, 62, 68). The cow (5, 7, 45, 46) and the hog (5, 7, 38, 39) have been used in four hematological experiments while the horse (5, 39, 40) and sheep (5, 51, 52) have been used in three similar experiments. These latter four animals are all large and, therefore, less convenient for experimental usage; however, the study of their reactions to selenium is important since they may develop selenium poisoning under natural conditions. The dog (2, 43, 48, 49) and the fish (9) have been used less frequently than the rat and the rabbit, although both are highly susceptible to selenium poisoning. The dog is par-

ticularly valuable in experimental studies because of the ease in obtaining large samples of blood.

In this laboratory we have found White Pekin ducks most satisfactory for experimental studies on the effect of selenium on hematopoiesis. It has been observed that sodium selenite interferes in some way with the maturation of erythrocytes. This apparently occurs within the bone marrow since the number of erythroblasts is decreased both within the peripheral blood and in the bone marrow of ducks given large doses of sodium selenite (50). Cysteine hydrochloride inhibits the effect produced by sodium selenite on the hematopoietic system (50).

Discussion. From this review of the hematological studies made upon animals poisoned by selenium, the lack of adequate studies on many phases of this problem becomes obvious. No references were found to the platelet counts either in acute or chronic selenium poisoning. There are conflicting opinions referable to the changes in the bone marrow. Little has been published relative to the effect of selenium on nucleated erythrocyts, although it is known that the hatchability of eggs is low and embryonic defects are high in chickens poisoned by selenium.

In view of the studies with radioactive selenium, it is possible that this isotope may prove to be a valuable tool for the study of the formation of erythrocytes.

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EFFECT OF CORTISONE ON EXPERIMENTAL RICKETTSIOSES AND ON ANTIBODY FORMATION*

DOROTHY M. WHITNEY AND LUDWIK ANIGSTEIN

The present study was prompted largely by the ever increasing interest in the mechanism of action of adrenal cortical hormones, particularly in their influence on the course of the infection, on antibody formation and immunity. In this regard the conflicting results obtained by various investigators indicate the complexity of the variables involved in the maintenance of the delicate balance between resistance and stress. There is however a concensus that although the cortical steroids alter the host's reaction to infection they do not affect the etiological agent directly (Kass *et al.*, 1950). Rickettsial infections were chosen for the present study inasmuch as no fundamental data as to their response to cortisone treatment are available.

Early studies on the relation of cortico-adrenal extract to immune processes have indicated stimulation in the response of the host (Fox and Whitehead, 1936). These observations were followed by Chase, White, and Daugherty (1946) who have found higher titers in antibody production in animals receiving adrenal cortical extract. Subsequent investigators were, however, unable to confirm enhancement of the immune reaction. On the contrary, the majority of observers have noted a lowering of antibody levels in laboratory animals sensitized with bacterial and viral antigens and treated with steroids.

It was shown that treatment with cortisone interfered with acquired immunity in abolishing the protective effect in mice of vaccination against *M. tuberculosis* (Solotorovski *et al.*, 1951). Cortisone may affect the course of virus infection as demonstrated for influenza A and B and mumps viruses in chick embryos (Kilbourne and Horsfall, 1951; Overman and Hanan, 1953), for poliomyelitis in hamsters (Schwartzman, 1952). The lowering of resistance to infection with otherwise

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non-pathogenic organisms normally present in rats treated with cortisone was shown by Selye (1951). The administration of cortisone during the primary attack of *Plasmodium cynomolgus* malaria in the rhesus monkey strikingly intensified the parasitic infection and provoked severe recrudescences (Schmidt and Squires, 1951).

In the light of these results the beneficial effect of cortisone when used either alone or in combination with specific chemotherapy in certain infections was rather unexpected. In cases of typhoid fever the combined therapy with cortisone and chloramphenicol provided more prompt relief than does chloramphenicol alone (Smadel *et al.*, 1951). Even when cortisone alone was used clinical manifestations were controlled. It is assumed that cortisone achieves its effect in assisting the host, since it has no direct effect on *Salmonella typhosa* (Woodward *et al.*, 1951). When cortisone was used as an adjunct to chloramphenicol in the treatment of Rocky Mountain spotted fever clinical improvement was rapid and dissipation of toxemia in the patient was apparent. Furthermore, the serological pattern in response to infection, namely a significant rise in titer of antibodies (Weil-Felix and complement fixation tests) excluded an interference of the therapy with antibody formation. (Workman *et al.*, 1952). Finally, a dramatic recovery of a moribund case of spotted fever treated with cortisone and aureomycin was recently reported (Arney, 1952). The rapid decrease of toxemia and fever was attributed largely to cortisone. This apparent antitoxic effect of cortisone could not be demonstrated in mice treated with the hormone and given toxins of virulent rickettsiae (Jackson and Smadel, 1950).

The purpose of the present study is to evaluate the role of cortisone in experimental rickettsioses, its effect on the clinical course, antibody levels and on the possible interference of this hormone with the immune responses of the host animal.

Material and Methods. Male guinea pigs of 500 to 600 gm. body-weight were used for experimental rickettsial infections and treatment with cortisone. During the course of infection and after recovery of the animals blood samples were drawn for determination of complement fixing antibodies. Modification of Bengston technique (overnight ice-box) was used. For the production of the specific antirickettsial sera male rabbits of 2.0–3.0 kg. were injected intravenously with epidemic typhus vaccine, Lederle, (0.2 cc. twice

weekly for 3 consecutive weeks) and bled 8 days after the last injection. Epidemic typhus, Breinl strain, and the Sheila-Smith strain of spotted fever both maintained in this laboratory were used as infective material for guinea pigs. Cortisone acetate (Cortone, Merck, or Cortogen, Schering) in concentration of 25 mg./cc. was injected subcutaneously into guinea pigs twice daily from 10–12.5 mg. per dose and 10 mg. daily intramuscularly into rabbits. The duration of cortisone treatment varied from 8 to 21 days.

Results. Whereas the guinea pig is known as the animal of choice for most rickettsial infections it has been reported as relatively resistant to cortisone treatment. In using 25 mg. cortisone per day for 8 consecutive days no visible deleterious effects in guinea pigs were noted. In other series of guinea pigs similarly treated over a period of 12 days a decline in body-weight up to 16 per cent was observed in some animals. Occasional deaths probably due to overdosage of cortisone were recorded by us, the autopsy showing congestion of the abdominal wall, abundant fat deposit in the abdominal cavity, small injected adrenal glands, injected testes and spleen either normal or reduced in size. Except for sporadic cases of toxic manifestations the response in the guinea pig corroborated the observations of others as to the resistance of this animal to cortisone and tolerance to massive dosage (Germuth *et al.*, 1952). This is also shown by observations that "cortisone, in large doses, does not lower the resistance of guinea pig to pneumococci. There have been reports however, that in guinea pig tuberculosis cortisone will cause the disease to spread so that at present it seems it is not possible to generalize on the effects of cortisone in the guinea pig" (H. J. Robinson, Merck Institute, personal communication). No untoward effects on rabbits treated with 10 mg. of cortisone daily over a period of 3 weeks were noted, however the milky appearance of the sera was striking. Cholesterol levels determined by Dr. E. E. Baird were found as 105 and 232 mg. per cent for 2 cortisone-treated rabbits as compared with 34 and 38 mg. per cent for 2 untreated rabbits. Similar observations were made by Knowlton (1952) in cortisone-injected rats in which cholesterol levels were consistently elevated as contrasted with normal, partially fasted or desoxycorticosterone treated animals.

The influence of cortisone on active immunization and antibody formation was studied in a series of 8 guinea pigs im-

munized against spotted fever with the specific vaccine. Four animals were injected once with a suspension of 0.5 cc. of vaccine in 0.5 cc. cortisone (12.5 mg.), whereas 4 guinea pigs were given one injection of vaccine only. The cortisone series was then treated for 11 days at 25 mg. per day per guinea pig. Blood samples were taken on the 11th day after vaccination was terminated and tested for complement fixing antibodies. Although complement fixation titers in all guinea pigs of this series were low (1:4 to 1:8), the challenge with spotted fever proved solid immunity of the cortisone series as well as of guinea pigs receiving a single dose of vaccine only. Furthermore, there was no appreciable difference in the complement fixation titers of either group. The experiments were continued in a series of 16 guinea pigs each of them injected twice, 6 days apart with spotted fever vaccine. Eight of this series were treated with cortisone (25 mg./guinea pig/day) for 12 consecutive days. Blood was collected on the 7th day following the last injection of vaccine. The complement fixation titers of the cortisone group were as follows: 1:16 in 1 case, 1:8 in 5 cases, and 1:4 in 2 cases. The average complement fixation titer was 1:8 in the cortisone series as compared with 1:13 of the non-cortisone group. The entire group of 16 guinea pigs was reinoculated with spotted fever 6 days after the second dose of vaccine and the treatment with cortisone continued for the next 5 days after infection. Of this series 3 guinea pigs died showing diarrhea, emaciation and subcutaneous hemorrhages. The recovered and reinoculated guinea pigs showed no symptoms of spotted fever thus indicating immunity.

These experiments indicate that immunity against spotted fever conferred to guinea pigs by active immunization with 1 or 2 injections of the specific vaccine (Lederle) was not influenced by cortisone although the antibody concentration in one series was lowered. It is to be noted that the complement fixation titers were generally low even in the vaccinated guinea pigs of the non-cortisone series, but this may be attributed to the vaccination limited to 1 or 2 injections. Nevertheless the guinea pigs became fully protected against reinoculation with virulent spotted fever. That cortisone may affect the immune response in vaccinated animals was shown by Solotorovsky and

associates (1951) in mice vaccinated against tuberculosis. The protective action of the vaccine was abolished by cortisone treatment.

TABLE 1

Antibody Response of Rabbits Immunized Against Epidemic Typhus Under Cortisone Treatment

Rabbit No.	Treatment	Complement fixation titers of sera during immunization 1st week	2nd week	3rd week
1	C	no data	160	160
2	C	no data	10	20
3	C	80	160	160
4	C	40	160	80
5	C	40	80	160
6	C	10	40	160
7	none	160	320	160
8	none	40	160	160
9	none	40	160	160
10	none	40	160	160
11	none	no data	160	160
12	none	no data	160	80
Normal rabbit serum		neg.	neg.	neg.
Human typhus serum		40	40	40

Figures represent endpoints of titration with complete fixation.
C—cortisone.

The influence of cortisone or experimental typhus was studied on a series of 7 guinea pigs pretreated with cortisone by injection of 12.5 mg. 24 hours before infection. The animals were then treated with 25 mg. cortisone daily for 11 consecutive days during which a high and continuous fever developed either the day following the infective dose or after an incubation period of 3–4 days. The duration of the febrile period was 10 to 14 days and in some guinea pigs as long as 20 days, exceeding the time of cortisone treatment. Although the course of the disease did not correspond to that of experimental typhus, the immunity of all the recovered guinea pigs to the subsequent challenge with typhus fever confirmed the diagnosis of typhus in the cortisone treated animals. In addition to the shortening of the incubation period, and prolongation of fever, a loss in body-weight was noted.

In another group of 6 guinea pigs cortisone treatment began simultaneously with typhus infection and was continued daily for 8 days (25 mg. per day in 2 divided doses). Only one ani-

mal showed a continuous high fever of 12 days duration after a short incubation period of 3 days thus indicating enhancement of the disease, while in the remaining animals of this series fever reactions were either typical or of an irregular type and the incubation period was variable. Although indications are that the course of typhus infection in the guinea pig can be altered by a prolonged action of cortisone, it would be safe to state that the clinical course of the infection depends on the physiological pattern of the guinea pig as a species as well as the individual variations in resistance.

In accepting cortisone as a factor interfering with resistance of the host to stress, an attempt was made to reactivate typhus fever in guinea pigs which have recovered from the disease. This experiment was planned to create conditions similar to those under which Brill's disease apparently occurs as a recrudescence of the primary infection with typhus. For this purpose 10 guinea pigs which recovered from epidemic typhus fever showing normal body temperature for 16 days were injected with 25 mg. cortisone daily for 8 days. An elevation of body temperature lasting for several days was recorded in 6 animals with 6 guinea pigs remaining afebrile. Although the nature of the secondary febrile reaction as a possible relapse of typhus fever was not clarified, there was no interference of the massive cortisone treatment with immunity as shown by the resistance of the entire series to a subsequent inoculation with typhus material. Investigations in this line are continued.

The response of the antibody formation to cortisone treatment was tested in rabbits sensitized with epidemic typhus rickettsiae, by 5 intravenous injections of 0.2 ml. typhus vaccine (Lederle) over a period of 14 days. Of a total of 12 rabbits 6 were injected during the vaccination with 10 mg. cortisone daily over a period of 21 days. Samples of blood were drawn at weekly intervals for complement fixation tests 3 times during immunization. The sera titers of the cortisone treated series were compared with those of the vaccinated nontreated controls.

Sera of all control rabbits showed a complement fixation titer 1:160 or higher by the end of the second week during vaccination, whereas only 3 of 6 cortisone-treated rabbits

showed a titer of 1:160, the titers of the 3 remaining rabbits of this series being 1:80, 1:40, and 1:10. Thus average titers for sera drawn 14 days after the first vaccine dose were 1:93 for the non-treated animals and 1:50 for the cortisone series. This apparently suppressive action of cortisone on the antibody formation becomes negligible during the third week of active immunization when the average titers for the non-treated and treated rabbits were 1:73 and 1:70, respectively. No differences were found in complement fixation titers at the end of the first week of immunization.

Summary and Discussion. It is suggested by this study that cortisone administration to guinea pigs and rabbits during active immunization with rickettsial antigens against spotted fever and typhus lowers the level of the antibody to either of the infections. However, considering the massive, subtoxic doses of cortisone administered intermittently during the entire period of sensitization, one may conclude that the suppression of antibody level is of low degree only. Similar partial suppression of antibody was recently reported by Overman and Hanan (1953) in rabbits vaccinated against mumps. If immunity can be expressed serologically by the amount of the antibody detected, then the low level of antibody was apparently adequate to protect the sensitized and cortisone-treated guinea pigs against multifold infective doses of spotted fever and typhus. At any rate, in no instance was there any evidence of interference between cortisone and acquired immunity to these rickettsioses. This applies to guinea pigs vaccinated against spotted fever even when receiving only one single injection of vaccine simultaneously with cortisone.

Rabbits subjected to hyperimmunization against epidemic typhus yielded antisera titers on higher levels than guinea pigs. The peak of antibody formation was reached at the end of the second vaccination week in the control group as compared with lower titers of the cortisone-treated series. The apparently enhancing effect of cortisone on the course of experimental typhus in guinea pigs was shown by shortening of the incubation period and prolongation of fever. The interpretation of the mechanism of action of cortisone on antibody formation still remains in the realm of speculation. In this regard the lowering of the

antibody level of the cortisone-treated host has been frequently emphasized as the primary function of cortisone responsible for the enhancement of infection. However, in applying this theory we have to exclude situations in which the embryonic stage of the host is involved and which due to immaturity is not provided with immunologic mechanisms. The enhanced concentration of viruses in chick embryos treated with cortisone deserves attention in this regard (Kilbourne and Horsfall, 1951). Other basic functions of cortisone such as influencing phagocytic activity, depressing the inflammatory response and possibly the reduction of the function of the reticuloendothelial system should be considered (Raffel, S., 1953) with the various steps in the formation of antibody, namely the assimilation of antigen, inflammatory reaction and the synthesis of antibody globulins (Fischel *et al.*, 1952). The cortisone attack on these various stages would largely depend on the relationship of the parasite to the host and on the nature of the antigen. The low degree of suppression of antibody to rickettsial antigens as shown in the present study would perhaps indicate an interference with local reactions mainly in the assimilation of the antigen rather than with synthesis of the antibody.

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Addendum

After this manuscript was submitted for publication, Aikawa and Harrell (*Proc. Soc. Exp. Biol. and Med.*, 83: 698–701, 1953) reported on the effect of cortisone acetate on experimental Rocky Mountain spotted fever in the guinea pig. It appeared that cortisone reduced the mortality rate of guinea pigs with moderately severe infections treated with 1 mg. cortisone starting on the day of infection with spotted fever and continued for 17 days. In another group of animals injected with 25 mg. of cortisone daily for 5 days at the peak of the illness, the fatality rate, during the period of administration, was signifi-

cantly lower than that in the untreated animals. However, according to the authors, the overall mortality rate was identical with that in animals not given the hormone.

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A METHOD FOR THE EVALUATION OF TUMOR GROWTH*

IRA R. TELFORD AND GEANE A. JEFFERY

In recent years the production of experimental lung tumors in mice has become a useful tool in the study of oncogenesis. The usual method of study consists of counting, under a low-power dissection microscope, the external lung tumors. (Heston, 1940; Shimkin, 1940; Telford, 1949; Shimkin and McClelland, 1949; Klein, 1952; Heston and Schneiderman, 1953). On the basis of this method a given experimental procedure is usually evaluated. Such a procedure is obviously inaccurate, since external counts do not indicate the total number or size of the numerous internal tumors, nor are they a true reflection of the total amount of lung tissue involved by the tumors. It would seem important therefore, that comprehensive data should be obtained on the total volume of the tumor masses in the lung before an accurate evaluation of any experimental procedure could be made.

We investigated the volume of tumor tissue in the lungs of A-strain mice by the following procedure: The lungs were prepared in the usual histological methods and cut serially at 50μ. The tissues were then lightly stained with hematoxylin. Using a micro-slide projector, tracings were made of the lung sections and each tumor was outlined on paper. We used two methods for securing the volume data. The first method consisted of making paper "cutouts" of each tumor outlined on the lung drawings. The weight of these "cutouts," compared with the total weight of the lung tracings, gave us the percentage by volume of lung tissue involved by tumors. This procedure, though fairly accurate, is laborious and time-consuming and was therefore abandoned in favor of the planimeter method. Employing a compensating polar planimeter, we carefully retraced the outlines of the tumors and thus we determined the

* From the Department of Anatomy, The University of Texas Dental Branch, Houston, Texas. This investigation was supported (in part) by research grants from the National Cancer Institute, U. S. Public Health Service. Received for publication May 25, 1953.

surface area occupied by the tumors in the lung. Since the thickness of the section is known, the total tumor volume can be calculated and the actual percentage of lung tissue occupied by the tumors can be determined. Comparison of similar data gathered by the planimeter method and data secured by the usual external tumor count method showed marked discrepancies (Table 1). It should be noted that on the basis of the

TABLE 1

Lung Tumors in Mice

Group	No. of Mice	Ave. number external tumors	Ave. number total tumors	Ave. total tumor vol. in mm.3	Ave. per cent of lung involved
1	48	36.4	56.0	294.57	7.27%
2	39	38.5	75.5	407.49	9.81%
Per cent difference		5.4%	25.8	27.7	25.6

average number of external tumors, there would appear to be little or no significant difference in the two groups. However, from the average total tumor volume data a significant difference between the groups appears. It is also of interest that with this particular set of data, there is an unusually close correlation between the average number of total tumors, average of total tumor volume and average per cent of lung involved. Such data suggests that total tumor counts may reflect the degree of lung involvement even though the size of the tumors is ignored.

For an accurate evaluation of an experimental procedure involving lung tumors, volumetric studies are indicated. External tumor counts, unsupported by total volume data or total tumor counts, are misleading and should be avoided. We suggest that the use of a compensating polar planimeter offers a convenient and accurate tool for obtaining such volumetric data.

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THE FORMULATION OF A GENETOTROPHIC SUPPLEMENT FOR THE EXPERIMENTAL TREATMENT OF DISEASES OF OBSCURE ETIOLOGY*

ROGER J. WILLIAMS AND LORENE L. ROGERS

Multiple vitamin and mineral therapy and prophylaxis in connection with numerous incompletely diagnosed conditions have become common practice, and the basis for this procedure, as well as the question of whether the currently used preparations are adequate to meet the required purposes, may well be examined in the light of present knowledge.

Until recently, the purpose of administering artificial assortments of vitamins and minerals has been based largely on the idea that many ailing individuals do not eat wisely and hence need supplements to aid them in rounding out the equivalent of a well-balanced food supply. Superimposed upon this consideration has been a more or less vague opinion that certain diseased conditions (infections) might call for an augmented supply of certain nutrients and that this probability might justify the use of vitamin and mineral supplements. Pregnancy also has received special consideration in this connection.

With the rise of the genetotrophic concept (Williams) a different rationale enters. According to this idea, individuals do not, for genetic reasons, have the same nutritional needs (quantitatively), and many diseases, the etiologies of which have hitherto been obscure, may have their origin wholly or partially in the deficiencies which arise because of individual demands which far exceed the average. Partial genetic blocks (Mitchell) or their effective equivalents, which are probably in the inheritance of all of us, must necessarily give rise to irregular (augmented) demands, which in individual cases may not be met by ordinary foods, even if wisely chosen from the standpoint of the needs of the general population.

Under a regimen in which the law of the "survival of the fittest" is allowed to operate freely, it is probable that the

* From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin, Texas.

possession of highly augmented nutritional demands would serve as an important mechanism whereby "unfit" individuals would be eliminated from a too rapidly expanding population. In present-day practice, however, we do not allow such considerations to obliterate our humanitarian impulses. We feel justified in prolonging the life and efficiency of "unfit" individuals, especially if this group happens to include ourselves and our friends.

The present and up-to-date purposes of administering vitamin and mineral supplements may therefore be three-fold: (1) to supplement foods unwisely chosen by individuals who develop ailments for this reason; (2) to supplement the needs of individuals whose medical histories (including pregnancy) demand it; (3) to supplement the diets of those who fare badly because of high inborn demands which are not easily met even by good food.

Ideally, of course, it would be desirable to determine for each treated individual the precise needs with respect to each nutrient, and to prescribe accordingly on an individual basis. Because of the multiplicity of known nutritional factors, the complexity of the considerations (including interrelationships), and the lack of tools for detecting and measuring deficiencies, no such procedure is at present practicable. Until such becomes possible, it is desirable to attempt, as has successfully been done in many cases of alcoholism (Williams, O'Malley, Smith), to furnish a balanced mixture of nutrients, which will with some reasonable degree of probability furnish the needed food elements to the ailing individuals in suitable amounts. Other nutrient elements that will not be needed in augmented amounts by the particular individual treated will, of course, be furnished at the same time.

If the genetotrophic concept is accepted, the basis for choosing supplements becomes somewhat altered. There is no *a priori* reason for thinking that one partial genetic block will be more common than any other, and deficiencies which arise for genetic reasons may involve relatively obscure nutrients as well as those which are better known by reason of their existence generally in foods in marginal amounts.

When exploratory investigations and trials are made in accordance with the genetotrophic concept on a disease of unknown etiology, it becomes desirable to administer a balanced mixture of nutrients in amounts such as to augment by several times the supply of these items as obtained in the food. From a practical standpoint, as will be clearer in our later discussion, *all* of the nutrients cannot be supplied in the form of supplements—many of them are far too bulky—so we will be concerned with those which can be supplied in capsules of convenient size and number and without inordinate expense. This unavoidably throws emphasis on what we may think of as "catalytic substances," such as vitamins and trace minerals. It should not by any means be presumed that explorations involving the genetotrophic concept should stop at this point, but it seems unavoidable that supplements which are to be used widely must have limitations with respect to bulk, weight, and cost.

It should be emphasized that the type of supplement with which we shall be concerned is definitely supplementary, and should not be considered as a substitute for food. Such supplements should, ideally, in order to accomplish their therapeutic purpose, be taken *along with* what is commonly considered a wholly adequate diet.

In table 1, columns 1 and 2, are listed all of the known nutrients which are probably essential in human nutrition, and the estimated average needs for each. Included are some items which could only be listed as "probable" so far as essentiality is concerned. The probability we have considered high when the need has been demonstrated in other mammals (which can readily be experimented with) but has not been specifically demonstrated in humans because of the lack of crucial experiments. Among the mineral elements we have included *boron*, for example, partly because there is a lack of contraindications regarding its use in supplements, and partly because its need by humans is at least remotely probable. The inclusion of *bromine* in this list is certainly based upon some uncertainty, but we have been led to include it, partly because it is widespread in living matter, has been isolated in a natural amino

acid, and appears to be selectively distributed in animal tissues. Other additional considerations (not by themselves weighty), are the facts that bromides have been used with benefit medicinally for a long time, and there are well-recognized idiosyncracies in response to this element. These facts suggest that some individuals may have an augmented nutritional need, and there are no indications that the amounts supplied by the suggested supplement could be detrimental.

TABLE 1

Human Nutritional Needs

Minerals	Estimated average daily adult need	"Unit Dosage" of items to be used	Fat Acids	Est. av. daily need	"Unit Dosage"
Calcium	0.75 g.	....	Linoleic acid (Linolenic, arachidonic, and vaccinic acids less effective)	1.5 g.	....
Phosphorus	0.75 g.	....			
Sodium	4 g.	....			
Potassium	2.5 g.	....			
Magnesium	0.25 g.	....			
Manganese	5 mg.	5 mg.	*Vitamins*		
Iron	10 mg.	10 mg.	A	5000 U	5000 U
Copper	2 mg.	2 mg.	D	400 U	400 U
Cobalt	0.1 mg.	0.05 mg.	E (α tocopherol)	6 mg.	6 mg.
Nickel(4)	1 mg.	....	K (menadione)	2 mg.	2 mg.
Zinc	12 mg.	9 mg.	C	50 mg.	2 mg.
Fluoride	0.5 mg.	....	Thiamin	1.5 mg.	1.5 mg.
Chloride	5 mg.	....	Riboflavin	1.8 mg.	1.8 mg.
Bromide	2 mg.	2 mg.	Niacin	15 mg.	15 mg.
Iodide	0.1 mg.	0.1 mg.	Pantothenic	10 mg.	10 mg.
Boron	5 mg.	2.5 mg.	Biotin	0.2 mg.	0.2 mg.
Molybdenum	0.5 mg.	0.2 mg.	Folic acid	1 mg.	1 mg.
Arsenic	0.3 mg.	....	B_6 group	3 mg.	3 mg.
Amino acids			B_{12}	2 μg.	2 μg.
L Tryptophan	0.25 g.	....	Paba	5 mg.	5 mg.
L Valine	0.80 g.	....			
L Phenylalanine	1.10 g.	....	Inositol	1 g.	....
L Lysine	0.80 g.	....			
L Methionine	1.10 g.	....	Choline	1 g.	....
L Threonine	0.50 g.	....			
L Leucine	1.10 g.	....	Rutin	5 mg.	5 mg.
L Isoleucine	0.70 g.	....			
L Histidine	0.5 g.	....			
L Arginine	1.2 g.	....			
L Glutamic acid	...	....			

In setting an "estimated average daily need," we have, where possible, used as bases the "Minimum Daily Requirements" of

the U. S. Food and Drug Administration, and the "Recommended Daily Allowances" of the Food and Nutrition Board of the National Research Council. We have, in general, chosen what seemed to us the most appropriate value. In the case of vitamin C we have selected a compromise value.

In the case of the great majority of items, however, we have had no such values to rely upon, and have necessarily estimated the average daily needs on the best of whatever other bases were available. We have taken the position previously taken by one of us that as a first approximation (lacking other information) one may assume (for the purposes that underlie this discussion) that the average nutritional needs of a population are about what that population would get if it consumed what nutritional science has determined to be a satisfactory, well-rounded diet. The amounts of several of the mineral elements and the lesser-known B vitamins indicated as "average daily needs" were determined in this manner, basic information having been obtained mostly from the excellent book *Trace Elements in Foods* by Monier-Williams and from Dr. Beerstecher's section of *Biochemistry of the B Vitamins*, A. C. S. Monograph 110. Excretion values were also considered in connection with estimating "average needs."

The values for most of the amino acid needs are those cited by Rose. The values for histidine and arginine, which may conceivably be needed by certain individuals, are those calculated by Harte and Travers from rat data. Glutamic acid is included in the list, without any amount being specified (the amounts present in proteins are often very high), to suggest its possible nutritional use in some cases, even though it is not thought to be a dietary essential and the need for supplemental amounts would presumably be relatively rare.

The estimated linoleic acid need (Burr) is based upon the need of 20 mg. per day by rats, a value which with admittedly meagre justification was multiplied by a factor of 75. This factor seems to be effective in several cases in translating rat needs into human needs, and should give values for our purposes at least of the right order of magnitude.

In the case of three vitamins—vitamin E, vitamin K, and rutin (vitamin P)—we have no adequate information with

regard to the amount in foods; so we have used 1/5 of what may be regarded a satisfactory therapeutic dose as the "estimated average need." This figure was chosen looking ahead to establishing a suitable level of use in the final nutritional supplement.

In column 3, table 1, we have listed the "unit dosage" of the various items which we think may well be included in the supplement. All items in which the weight of the "unit dosage" would be 0.2 g. or more are omitted from the list because they are too bulky to be given in capsule form with reasonable convenience. This excludes such elements as calcium, phosphorus, sodium, potassium and magnesium, as well as all the amino acids and linoleic acid, inositol, and choline.

This does not mean that the genetotrophic concept should be forgotten in connection with these items, but rather that these items cannot with practicality be included, *in sufficient amounts*, in a genetotrophic supplement for wide use. In specific cases where the facts seem to justify the trials, any of the omitted items, at suitable levels, may well be used.

One of the questions to be considered (especially is this true in the case of the trace elements) is whether the margin between the amounts to be used (3–9 "unit dosages" per day) and the toxic level is sufficiently wide to be perfectly safe (Monier-Williams). *Manganese* is relatively non-toxic; the critical limit of consumption is thought to be about 200 mg. per kg. of body weight. This would mean 14 g. daily for a 70 kg. man, which is very far above the 45 mg. maximum obtained from 9 "unit dosages". *Iron* offers no problem since it is non-toxic and is not readily absorbed. The dosage of *copper* needs to be scrutinized. Sheep are said to be very susceptible to poisoning by it; yet it requires 1 gram of copper sulfate daily for one or two months to kill them. On this basis, 12 mg. of copper per day, even when used on a continuous basis, or 18 mg. daily for a shorter period of time, should be safe for humans. The fact that liver contains (as sold) 50 p.p.m. and oysters up to 400 p.p.m., is an indication of the safeness of a much higher copper intake than our supplement provides. *Cobalt* is relatively toxic. For small animals the lethal dose of cobaltous oxide is 0.01 to 0.14 g./kg. of body weight. For dogs it is 0.03 g./kg. For man, the lethal dose is calculated to be about 2 g. Our supplement on the basis of 9 "unit dosages" supplied 0.45 mg. About 0.1 mg. daily is known to be required by sheep. *Nickel* is not highly toxic, but the need for it does not seem probable, so it is omitted. *Zinc* is relatively non-toxic. A dog was given nearly a half gram of Zn daily for a year without apparent harm. From 12 to 120 g. of zinc as zinc lactate was given daily to brood mares for 2½ years without

toxic symptoms. Our supplement at the maximum 9 "unit dosages" level furnishes 81 mg. The margin between *fluoride* requirement and the toxic level appears to us to be too narrow to make safe its introduction in a genetotrophic supplement for wide use. *Bromide* is relatively non-toxic and the small amount supplied by our supplement appears perfectly safe. A similar statement holds for *iodide*. *Boron* in the form of boric acid (or borax) is relatively non-toxic, as is indicated by the fact that in humans 0.5 to 1 g. daily (90 mg. to 180 mg. boron) is required to interfere with absorption of food and induce loss of weight. In Britain, 0.4 per cent boric acid was allowed in cream as a preservative. However, due to the fact that some individuals appear to show idiosyncracies and to have some distress from relatively small doses, we have reduced the *maximum* level of boron to 22.5 mg. daily. *Molybdenum* shows toxicity to cattle when the content is 20–200 p.p.m. in the dry matter of the pasturage. At 5 p.p.m., however, the toxicity is absent. Assimilable food containing 5 p.p.m. would give, in a human diet, about 2.5 mg. daily of molybdenum. This corresponds to more than the maximum amount which 9 "unit dosages" would yield. The inclusion of molybdenum in a supplement is made more desirable by the recent finding (De Renzo) that it functions in an animal enzyme system. In the case of *arsenic*, the margin between the amount needed, if any, and the amounts that may cause toxicity in highly susceptible individuals is too narrow to make its inclusion safe.

It is to be emphasized that, although multiple supplements are called for in accordance with the genetotrophic concept, a supplement should *not* be judged solely by the *number* of items it contains, because in many such supplements, dietary factors have been added in wholly insignificant amounts. To cite extreme cases (taken from supplements which are on the market and generally to be rated superior) we have noted one widely used preparation which contains in the recommended dosage 18 mg. of iron and 0.36 mg. of manganese. In view of the fact that many foods contain as much as 10–30 p.p.m. of manganese, and some contain 100 or more p.p.m., the amount of manganese in this supplement is ridiculously low. It is about the amount present in one tablespoonful of rolled oats.

Still other preparations contain a few milligrams of calcium and phosphate—amounts that are wholly insignificant. It is rather common practice to put into mineral supplements 1 mg. each of manganese and zinc. It seems to us that these items should either be left out entirely, or else put in at levels which will contribute substantially to the total supply present in the food.

In the field of the vitamins, the discrepancies between the relative amounts furnished by standard preparations which are on the market and the amounts present in diets are often almost as striking. In one common and supposedly reputable preparation, the thiamin-folic acid ratio is 100:1 (10 mg. thiamin, 0.1 mg. folic acid), whereas actually the amounts of the two vitamins needed are of the same order of magnitude. In another product, the amounts of thiamin, calcium pantothenate, folic acid are respectively 15 mg., 3 mg., 0.1 mg. (ratio 100: 20: 0.7), whereas, according to our study, these vitamins should be present in about the ratio 100: 700: 70.

Insignificant amounts of amino acids (methionine, for example), inositol, choline and other dietary essentials, are often included in dietary supplements. The public should, in some manner, be shielded against the form of deception which involves listing minerals and vitamins as ingredients of a food supplement, when the amounts present are entirely too small to be significant. The addition of extracts of liver, yeast, wheat germ, alfalfa, watercress, etc., to vitamin and mineral supplements may extend the list of the nutritional factors present (even to include unknown factors), but it seems certain that no such extract, under practical conditions, can contain enough of anything, to make an substantial contribution to the value of a supplement. The basic reason for this has been given in an earlier publication.

We have not considered the problem, which is primarily but not wholly one in pharmacy, of exactly the best form in which to introduce the various trace elements and vitamins. In the case of trace elements where there is the slightest danger of toxicity, this problem may be important. Neither have we gone into the problem of how large the capsules should be or how many "unit dosages" (whether one or more) should be placed in each capsule. These are questions which manufacturers and distributors will answer for themselves, and on which we have no expert opinions. It may be desirable both from the standpoint of practical use, and manufacture, to have the mineral supplement separate from the organic supplements.

Because individuals sometimes show unlooked-for idiosyncracies, we believe it is desirable to start a genetotrophic sup-

plement such as we have suggested, at a relatively low level of intake, perhaps three "unit dosages" per day, and to increase the dosage as may be needed or indicated by the particular case under trial. It is rather important, we believe, that supplements such as this one be taken with meals distributed throughout the day. Assimilation will be better, and the supplement will be utilized when and where needed, if it is allowed to mix with regular food instead of being taken in concentrated form only once a day.

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